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FACULTAD DE CIENCIAS DE LA SALUD

Diseño de nuevas bebidas funcionales enriquecidas en fitoquímicos bioactivos

Design of new functional beverages rich in bioactive phytochemicals

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Directores:
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Murcia, 26 de Junio de 2014

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1. **Gironés-Vilaplana, A.**, Valentão, P., Andrade, P.B., Ferreres, F., García-Viguera, C., Moreno, D.A. "Quality and antioxidant capacity of novel beverages based on lemon and exotic berries" 2012 In: Proceedings of 6th Central European Congress of Food. Eds: Nedovic V., Ilic N., Tumbas V., Kalusevic A. pp 7-13. ISBN: 978-86-7994-027-8.

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5. **Gironés-Vilaplana, A**., Moreno, D.A., García-Viguera, C. "Identification and quantification of anthocyanins in traditional 'patxarán' and in new maqui berry liquor". 7th International Workshop on Anthocyanins. Oporto (Portugal), 2013.

6. **Gironés-Vilaplana, A.**, Baenas, N., Villaño, D., Speisky, H., García-Viguera, C., Moreno, D.A. "Evaluation of Phytochemicals and Biochemical Activity of Native Iberian-American Fruits". 1st European Conference on Natural Products: Research and Applications. Frankfurt (Germany), 2013.

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1. **Gironés-Vilaplana, A.** International seminar: "Diseño y caracterización de nuevas bebidas funcionales de zumo de limón con frutos ricas en compuestos bioactivos". Seminario Internacional Seguridad, Calidad y Funcionalidad de los Alimentos: Retos de la innovación alimentaria en un mercado global. Medellín (Colombia), 2014.

"La verdadera ciencia enseña, por encima de todo, a dudar y a ser ignorante"

Miguel de Unamuno

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ABBREVIATURES

AA	Ascorbic acid
ABTS$^+$	2,2-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt
AChE	Acetylcholinesterase
AD	Alzheimer disease
BuChE	Butirilcholinesterase
DHAA	Dehydroascorbic acid
DNA	Deoxyribonucleic acid
DPPH$^\cdot$	2,2-diphenyl-1-picrylhidracyl radical
EFSA	European Food Safety Agency
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FRAP	Ferric reducing antioxidant power
HOCl	Hypochlorous acid
HPLD-DAD	High Performance Liquid Cromatography with Diode Array Detection
NOS	Nitric Oxide Synthase
$O_2^{\cdot-}$	Superoxide radical
$^\cdot$OH	Hydroxyl radical
ORAC-Fl	Oxygen radical absorbance capacity
RC	Relative concentration
RDA	Recommended Dietary Allowance
ROS	Reactive Oxygen Species
TA	Total acidity
TSS	Total soluble solids
UV	Ultraviolet light
WHO	World Health Organization

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CHAPTER 1. INTRODUCTION

1. BACKGROUND AND TRENDS IN PLANT DERIVED-FOODS AND HEALTH

More than two thousand years ago, Hippocrates, the precursor of medicine, coined the aphorism '*Let your food be thy medicine and medicine be thy food*' showing that the curative properties of food are known since ancient times. Nevertheless, only recently a body of scientific evidence supporting the relationship between food and health has emerged (Dillard & Bruce German, 2000), and increasing interest in society for a complete and healthy diet, in which fruits and vegetables, (Schröder, 2007), play a key role (Figure 1.1). An estimated 80% of cardiovascular diseases, 90% of Type II Diabetes (non-insulin-dependent) and a third part of cancers could be avoided including changes in lifestyle and dietary improvement (Gil & Tomás-Barberán, 2008), contributing fruits and vegetables to the prevention of these diseases (Mullen *et al.*, 2007).

Figure 1.1. New consumer trends to healthy, easy to prepare, and safe foods.

The evolution of the agrifood sector is linked to market trends, being the objective satisfying the final consumers, either national or international. While fruits and vegetables are usually consumed fresh, a large proportion need to be processed and/or preserved due to economic and logistical reasons, such as improve digestibility, culinary needs and enhance the intake of certain consumer

groups (children, elderly, sick, or people with limited time to prepare food, etc.). In general, the European population does not consume the minimum amount recommended of fruits and vegetables, not being Spain an exception. Perhaps only taking into account the juice consumption the minimum could be reached, reason why it is necessary to ensure the nutritional content of these drinks (Seeram *et al.*, 2008).

The growing interest in the study of natural antioxidant compounds has been accompanied by a rapid increase in the known as "functional foods" market, where fruit and vegetables have a predominant role. The food industry, aware of scientific advances, can present their products with added value, becoming more and more accepted by consumer, with better nutritional and organoleptic characteristics (Gruenwald, 2009; Ozen *et al.*, 2012).

(1.1.) FUNCTIONAL FOODS AND BEVERAGES

"Functional foods" are those foods that are made not only for their nutritional characteristics, but also to perform a specific benefit beyond nutrition. Because of this, certain biologically active components are added, such as minerals, vitamins, fatty acids, dietary fiber or antioxidants, etc. (O. J. E. U. Regulation (EC) No. 1924/2006 on nutrition and health claims made on food). This operation of adding exogenous nutrients is also called "fortification". This type of fortified food is an emerging field of food science and technology with potential in research in the food, nutrition and health area. Among the outstanding achievements, in the scientific literature and in the marketing of food products, we can found the improving of gastrointestinal functions, the contribution of redox and antioxidant systems as well as the modification of the macronutrients metabolism (Roberfroid, 2000).

Growth of the global markets of functional foods now appears to be slowing down, due to factors such as the economic recession and the fact that various authorities (i.e. EFSA, FDA) have started to impose limits upon the health claims. Nevertheless, new products development remain reasonably high, and health related product trends observed, throughout the developed world (such as rising obesity rates), suggest that the potential market for many types of functional foods remains positive. Besides, the production of this type of functional foods is

represented by three main countries/continents (Japan, USA and Europe) (Figure 1.2) (Leatherhead Food Research, 2011).

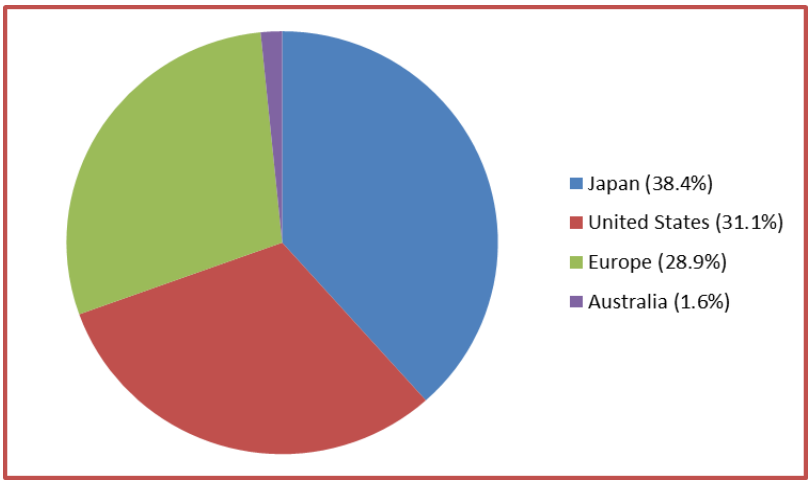


Figure 1.2. Global functional foods market by regions in 2010 (% value). Source: Leatherhead Food Research

The design of fruit beverages, rich in bioavailable and bioactive compounds, can be the basis of new functional foods with potential health benefits, due to the close relationship between a physiological positive state of oxidative stress as a trigger for different health problems (cardiovascular, metabolism of glucose and lipids, neuronal activity, anxiety, etc.). The growing interest in new added-value foods and beverages with health-promoting properties has led to the development of new beverages based on different kinds of waters, juices and non-alcoholic drinks enriched with fruits, as source of nutrients and bioactive compounds, being the beverages section an important portion of the global functional market (Figure 1.3).

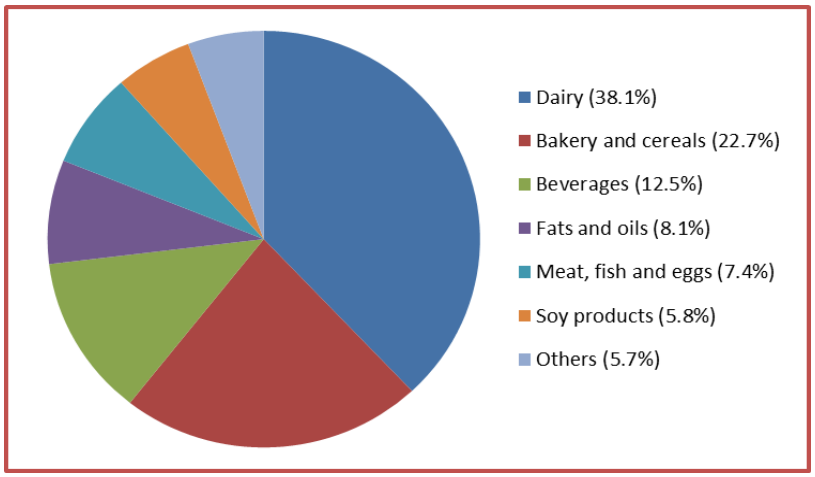


Figure 1.3. Global functional foods market by sector in 2010 (% value). Source: Leatherhead Food Research

The choices for beverages have become so specific that they appear to be almost tailored for an individual, representing an extension of one's personality. Consumers have their choice of beverages that aid in boosting energy, shrinking waistlines, sharpening mental focus, preventing pain associated with bone and joint conditions, and the list goes on. In addition, there are beverages that are specific for each age demographic and gender, with a growing focus on products targeting kids, women and seniors. This diversification in beverages in conjunction with the increased channels in distribution continues to fuel consumer demand.

The functional beverage market has increased steadily over the past decade, with a sharper rise in the last few years. According to Datamonitor, the global non-alcoholic beverage market is valued at just under $500 billion worldwide, with Europe accounting for a large portion: $189 billion (Fortitech, 2012) (Figure 1.4).

Over 80% of this market can be attributed to sales from North America, Western Europe and Asia Pacific.

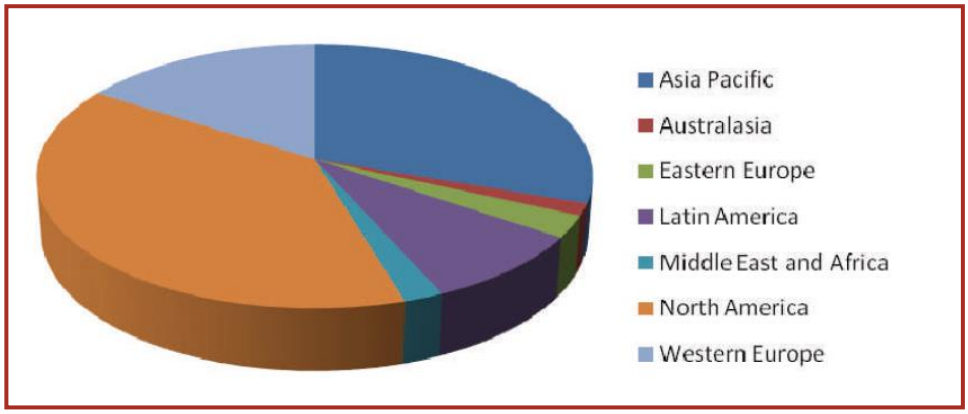


Figure 1.4. Worldwide fortified beverage sales by region (2009). Source: Euromonitor.

In this sense, among the different vegetable products available in Murcia Region (Spain) and in the entire eastern part of the Iberian Peninsula, highlights the lemon production. Several studies have reported that lemon is an important source of nutrients and phytochemicals, including flavonoids, citric acid, vitamin C, and minerals (González-Molina *et al.*, 2008b), with numerous beneficial health promoting properties (Adibelli *et al.*, 2009; González-Molina *et al.*, 2010), as will be explained ahead. For this reason, lemon juice is an interesting food matrix for designing new beverages, as well as being a suitable source of value-added products since overproduction and non-marketable fruits lead to a serious environmental problem of unused agrowaste on a yearly basis. Due to this, lemon juice represents an alternative for the conversion of a bioburden into a food product.

In the same way, red fruits (berries) are rich in phenolic compounds, particularly anthocyanins, responsible for their colour; and depending on the species, ellagic acid derivatives, vitamin C, and other phytochemicals (Del Rio *et al.*, 2010). Recent studies have shown that berries have beneficial effects against cancer (Schreckinger, Lotton, *et al.*, 2010), cardiovascular (Basu *et al.*, 2010), and neurodegenerative diseases (Shukitt-Hale *et al.*, 2008) among others, attributed to these bioactive compounds. Some of these bioactive and understudied berries are maqui (*Aristotelia chilensis*), açaí (*Euterpe oleracea*), and sloe (*Prunus spinosa*), which will be detailed later in this section.

Previous research has shown that combining lemon juice (*Citrus limon* (L.) Burm. f.) with berry concentrates and powders improves the organoleptic

characteristics, and the biological activity of the final product by means of antioxidant capacity and enzyme modulation (González-Molina *et al.*, 2008a) (González-Molina *et al.*, 2012), offering new possibilities for new products to support nutrition and public health problems associated with non-communicable diseases, on adult population (i.e. obesity, Diabetes Mellitus, etc.).

(1.2.) FIRST WORLD DISEASES

Currently, more than 55% of the Spanish adult population is overweight (National Health Survey) and in the UE nearly 60% of adults, representing almost 260 million adults, are overweight or obese (WHO, 2011) being expected that in 2015, 2.3 billion adults will be overweight and 700 million will be obese in worldwide (globesity or obesity pandemic) (Berghöfer *et al.*, 2008; Doak *et al.*, 2012). Obesity is increasing in countries of high, medium and low life-quality and with negative personal and public health consequences. Moreover, obesity is accompanied by numerous diseases, so a global effort to monitor and treat this pandemic is necessary.

This increasing trend in obesity in the first world is accompanied by a growing incidence of diabetes. The close relationship between these two diseases has led to the adoption of term *diabesity* (Schröder, 2007). In this aspect, the inhibition of α-glucosidase, a key enzyme that catalyses the final step in the digestive process of carbohydrates, could delay the digestion of oligosaccharides and disaccharides to monosaccharides, diminishing glucose absorption and consequently reducing prostprandial hyperglycemia (Rubilar *et al.*, 2011). For that, natural α-glucosidase inhibitors have been searched, being reported berry polyphenols as effective inhibitors (Boath *et al.*, 2012). On the other hand, the current therapeutic approaches for the treatment of obesity involve the inhibition of dietary triglyceride absorption via inhibition of pancreatic lipase (PL) by orlistat (Birari & Bhutani, 2007). Many polyphenolic extracts are active against this enzyme, like from grape seeds (Moreno *et al.*, 2003), or from certain berries, described as effective inhibitors of pancreatic lipase (McDougall *et al.*, 2009).

In this sense, the loss of basal forebrain cholinergic cells in adult population results an important reduction in acetylcholine, which is believed to play an important role in the cognitive impairment associated with Alzheimer's disease

(AD), senile dementia, ataxia, myasthenia gravis and Parkinson's disease (Mukherjee *et al.*, 2007), also important diseases of elderly and aging adults in first world. Taking into account that cholinesterases, such as acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) are the principal enzymes involved in the hydrolysis of acetylcholine, cholinesterase inhibitors are being developed for the treatment of these diseases. In addition, a wide range of plant compounds with cholinesterase inhibitory activity have been found that may be relevant to the treatment of these neurodegenerative disorders (Mukherjee *et al.*, 2007). Some phenolic compounds have been described as cholinesterase inhibitors (Brühlmann *et al.*, 2004; Khan *et al.*, 2009), and in other research rodent models revealed that the polyphenolic compounds found in some berries may decrease the risk of developing age-related neurodegenerative diseases (Shukitt-Hale *et al.*, 2008).

2. PHENOLIC COMPOUNDS

Phenolic compounds are plant secondary metabolites commonly found in fruits and vegetables with attributed pharmacological properties (Parr & Bolwell, 2000). They are essential to plant's physiology, playing a key role in diverse and necessary functions such as structure, pigmentation, pathogen and predator resistance, growth and development (Croteau *et al.*, 2000) (Table 2.1).

Table 2.1. Classification of phenolic compounds.

FLAVONOIDS		NON-FLAVONOIDS
Chalcones	Flavanones	Phenols
Aurones	Flavones	Benzoic acids derivatives
Isoflavones	Flavonols	Cinnamic acid derivatives
Flavan-3-ols		Ellagitannins
Flavan-3,4-diols		Gallotannins
Proanthocyanidins		Phlorotannins
Anthocyanins		Stilbenes

Phenolics are characterized by having at least one aromatic ring with one or more hydroxyl groups attached, and range from simple, low molecular weight, single aromatic-ring compounds to the large and complex tannins and derived polyphenols (Crozier *et al.*, 2009). They are commonly found conjugated to sugars and organic acids and can be classified into two groups, the flavonoids and the non-flavonoids.

(2.1.) FLAVONOIDS

Flavonoids are one of the most widespread groups of secondary plant metabolites (Robards *et al.*, 1999), present in a wide variety of edible fruits and vegetables. Flavonoid skeleton is composed of two aromatic rings (namely, A and B), which are connected through a pyrone or hydropyrone ring (C)(Gattuso *et al.*, 2007). The main subclasses of these C6–C3–C6 compounds are the flavones, flavonols, flavan-3-ols, isoflavones, flavanones, and anthocyanidins. Other flavonoid groups are the chalcones, dihydrochalcones, dihydroflavonols, flavan-3,4-diols, coumarins, and aurones (Figure 2.1).

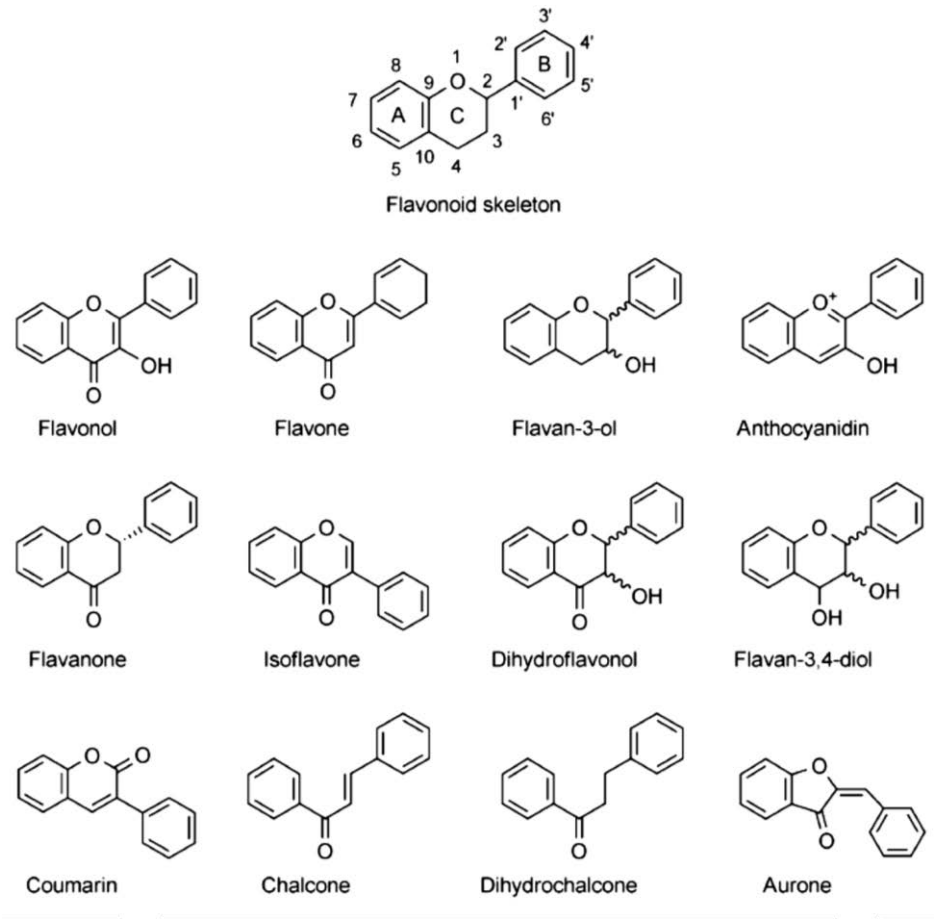


Figure 2.1. Flavonoids. Skeleton and types. Extracted from Crozier *et al.*, 2009.

The basic flavonoid skeleton can have numerous substituents. The majority of flavonoids occur naturally as glycosides rather than aglycones (Del Rio *et al.*, 2013). In this section we will deepen about the major flavonoids of the studied fruits.

2.1.1. Flavanones

Flavanones are the most abundant *Citrus* flavonoids. These compounds can be easily converted to isomeric chalcones in alkaline media (or vice versa in acidic media) provided that there is a hydroxyl substituent at position 2' (or 6') of the chalcone (Tomás-Barberán & Clifford, 2000). Their chemical structures are almost

specific for every species, something that can be used as tool for adulteration detection purposes in commercial juices (Calabrò *et al.*, 2004). Flavanones exist as a pair of diastereomers because of the presence of a chiral centre in the aglycone (C-2) and the optically active sugar residue. Naturally occurring flavanones usually have the 2S configuration, but racemization can take place during extraction (Tomás-Barberán *et al.*, 2000) (Figure 2.2).

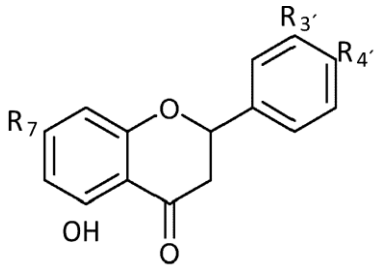


Figure 2.2. General flavanones structure

2.1.2. Flavones

Flavones are not distributed widely, and only have been reported with significant occurrences in celery, parsley, some herbs and *Citrus* species. Flavones lack oxygenation at C3 but otherwise may have a wide range of substitutions including hydroxylation, methylation, *O*- and *C*- alkylation and glycosylation. Most flavones occur as 7-*O*- glycosides (Crozier *et al.*, 2009).

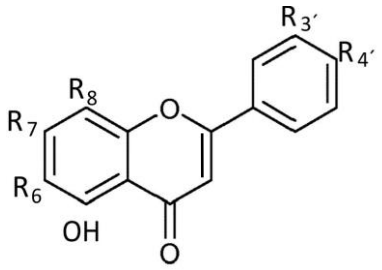


Figure 2.3. General flavones structure

2.1.3. Flavonols

The flavonols are the most widespread of the flavonoids, being dispersed throughout the plant kingdom with the exception of algae (Crozier *et al.*, 2009). They have substitution patterns commonly involving A and/or B ring with hydroxylation in the 5 and 7 or 3′ and 4′ positions, respectively (Herrmann, 1976) (Figure 2.4).

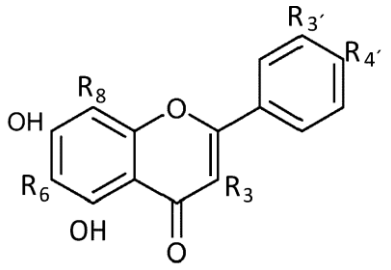


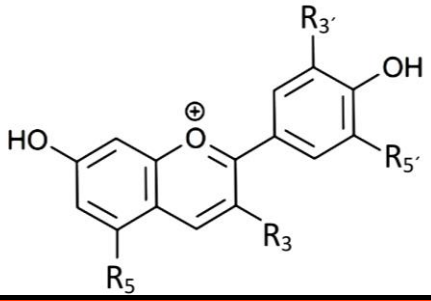
Figure 2.4. General flavonols structure

The flavonols, quercetin, kaempferol, myricetin and isorhamnetin, predominant in fruits, are most commonly found as *O*-glycosides or, less commonly, as *C*-glycosides, in which one or more of the phenolic hydroxyl groups is bound to a sugar or sugars by an acid-labile hemiacetal bond.

2.1.4. Anthocyanins

Anthocyanins comprise the largest group of water soluble pigments in the plant kingdom and are especially characteristic of the angiosperms or flowering plants, which themselves provide our major source of food crops. In food plants, anthocyanins are widespread occurring in at least 27 families, 73 genera and a multitude of species (Bridle & Timberlake, 1997). Anthocyanins occur naturally in plants in the form of glycosides in which the anthocyanidin molecule is coupled with a sugar. The part of the pigment that exists free of sugar (generically known as aglycone) is called anthocyanidin. Anthocyanins can be classified into different types based on modifications, such as substituent groups on the B ring, type and number conjugated sugar, and the presence or absence of an acyl group. There

are at least six principal types of anthocyanins: pelargonidins, cyanidins, delphinidins, peonidins, petunidins, and malvidins (Figure 2.5).



Antocyanin	Abbreviature	$R_{3'}$	$R_{5'}$
Pelargonidin	Pg	H	H
Cyanidin	Cy	OH	H
Delphinidin	Dp	OH	OH
Peonidin	Pn	OCH_3	H
Petunidin	Pt	OCH_3	OH
Malvidin	Mv	OCH_3	OCH_3

Figure 2.5. Main anthocyanins structure

This group of naturally occurring pigments is of growing interest, not only for technological reasons and due to their organoleptic properties but also because of their potential health-promoting effects, as suggested by the available experimental and epidemiological evidence (Castañeda-Ovando *et al.*, 2009; de Pascual-Teresa *et al.*, 2010; Tsuda, 2012).

Nevertheless, anthocyanins are rather unstable compounds, being influenced by pH, oxygen, light, temperature, concentration, enzymes, as well as presence of ascorbic acid, sugars, metal ions, sulphur compounds, and copigments (Castañeda-Ovando *et al.*, 2009; García-Viguera & Bridle, 1999; Jurd, 1967; Parisa *et al.*, 2007).

2.1.4.1. pH

Anthocyanins exist in different chemicals forms, both coloured and colorless, according to pH. In acidic or neutral media, four anthocyanins structures can be presented in equilibrium: the flavylum cation (AH^+), the quinoidal base, the carbinol pseudobase and the chalcone (Figure 2.6).

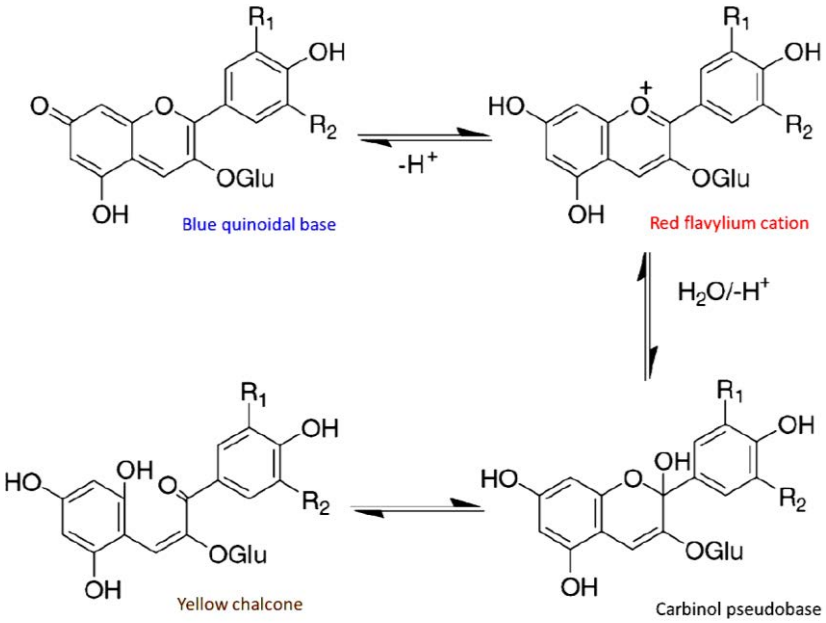


Figure 2.6. Chemical structures of anthocyanins depending on pH.

At pH 1-3 the red flavylum cation is the most abundant chemical form. The increasing of pH is accompanied by a rapid loss of a proton generating the blue quinoidal base. Consequently, a much slower hydration of the flavylium cation happens to yield the colourless carbinol pseudo-base that tautomerises through an opening of the C-ring to generate the yellow chalcone.

2.1.4.2. Copigmentation

Copigmentation is one of the most important factors leading to the profuse colour variability observed in flowers and fruits and it is also supposed to account for a variable part of the colour of red wines (Timberlake & Bridle, 1976).

Copigmentation can occur through several interactions, i.e. intramolecular, which an organic acid, an aromatic acyl group, or a flavonoid (or some combination thereof) is covalently linked to an anthocyanin chromophore, or through loose intermolecular interactions, which colourless flavonoids or other phenolic compounds interact through weak hydrophobic forces with anthocyanins. Self-association and metal complexation are also possible means through which copigmentation occurs (Castañeda-Ovando *et al.*, 2009) (Sari *et al.*, 2012). The colour stability of an anthocyanin-solution can be enhanced when the concentration of anthocyanins increase, forming copigments with flavonoids (own anthocyanins or others), organic acids, metals, or other anthocyanins (Boulton, 2001; Castañeda-Ovando *et al.*, 2009). Numerous studies have been carried out in order to increase the knowledge of anthocyanin copigmentations during storage in berry products and wines (González-Manzano *et al.*, 2009; Malien-Aubert *et al.*, 2001; Rein & Heinonen, 2004).

2.1.1.4.3. Others

Compounds such as ascorbic acid, sulphur compounds or enzymes (polyphenoloxidase, peroxidase, glycolases and esterases) may account for anthocyanin degradation (Brownmiller *et al.*, 2008; De Rosso & Mercadante, 2007; García-Viguera *et al.*, 1999). Therefore, increases of temperature, light effect, direct oxidation and metal irons can also affect the anthocyanin stability (Castañeda-Ovando *et al.*, 2009; Turker *et al.*, 2004).

(2.2.) NON-FLAVONOIDS/PHENOLIC ACIDS

Within the non-flavonoid phenolic compounds, highlight the phenolic acids that can be classified into two broad groups: benzoic acid derivatives and hydroxycinnamic acid derivatives, being this last group the most common in nature (Claudine Manach *et al.*, 2004) (Figure 2.7).

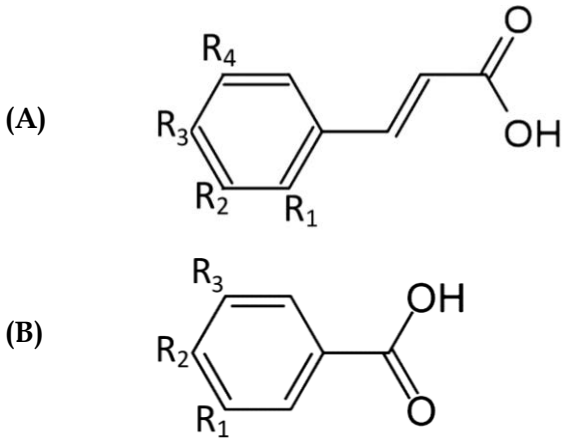


Figure 2.7. General structure of phenolic acids: hydroxycinnamic (A) and benzoic (B) acids.

3. LEMON

(3.1.) ORIGIN, VARIETIES, AND GLOBAL AND NATIONAL PRODUCTION

Although the exact genetic origin of cultivated *Citrus limon* is not clear, this fruit is original from Asia, being reported for the first time at third or fourth century A.C. (Figure 3.1).

Figure 3.1. *Citrus limon*: Tree, flower and fruit

The Spanish production is essentially based on two native varieties: Fino and Verna, representing over 95% of total production.

Citrus fruits are among the most important horticultural crops (131.3 x 10^6 mT in 2012), and are consumed mostly as fresh product or juice because of its nutritional value and special flavour. Total *Citrus* production in Spain was 5.5 X 10^6 mT in 2012 (Faostat, 2012), being the sixth producing country in the world after China, Brazil, U.S., India, and México (Figure 3.2).

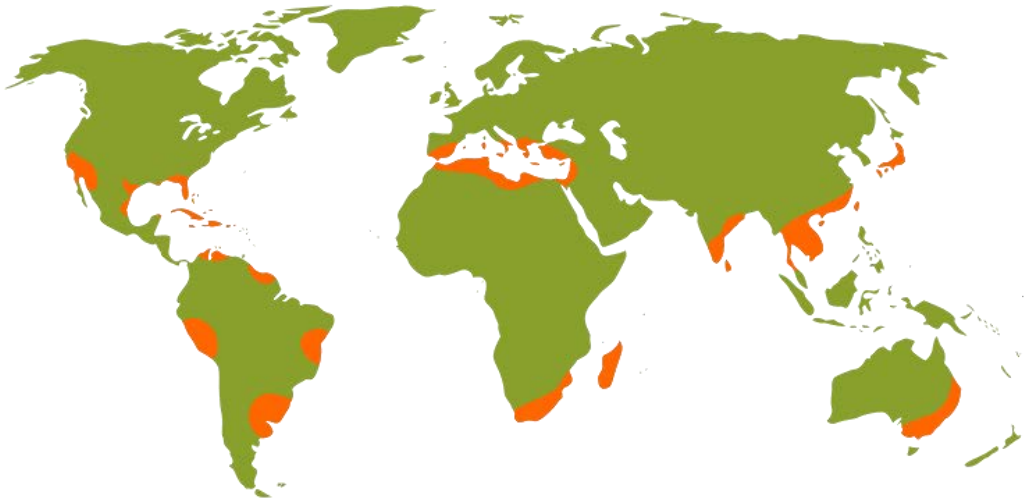


Figure 3.2. Major *Citrus* growing regions according to Weltenburger 1987 with updated data from 2010.

The majority of the *Citrus* production in Spain is accounted for oranges (*Citrus sinensis* L.), a significant quantities of mandarins (*Citrus reticulata* Blanco), lemons (*Citrus limon* Burm. f), limes (*Citrus aurantifolia* Christm.), and grapefruits (*Citrus paradisi*, Macfad) (Figure 3.3).

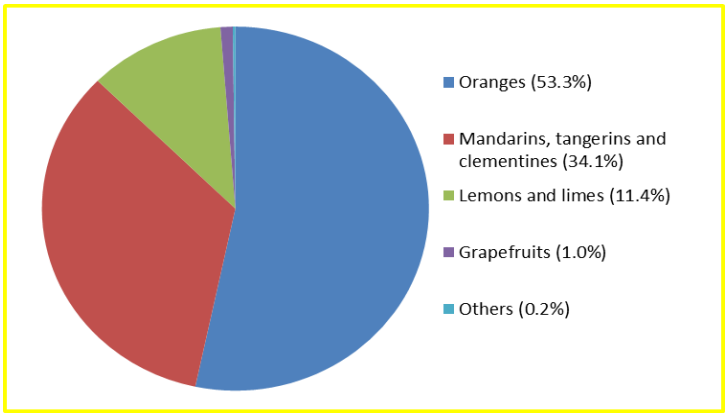


Figure 3.3. Spanish *Citrus* production in 2012. Source: FAOSTAT.

According to FAO data, total world lemon production was 13.9 x 10^6 mT in 2011, being Spain (773.620 tonnes) the eighth producing country in the world (UN Food & Agriculture Organization, 2011) (Table 3.1).

Table 3.1. *Citrus* production (tonnes) in Murcia Region in recent years.

Citrus fruits	2008	2009	2010	2011*	2012*	2013[a]
Lemon	390000	263875	402316	401935	356210	390000
Mandarin	57000	56825	54258	72022	71507	88900
Orange	185026	150514	167292	148768	144967	169400
Grapefruit	22960	23330	28478	31630	33280	31220
Others	-	810	610	610	520	580
TOTAL	654986	495354	652954	654986	606484	680100

*Provisional data. [a]Estimated data. Source: CARM

Spain is the Europe leader producer of lemons and specifically in southeastern Spain, where Murcia is leading with 50% of national production, followed by Alicante (30%), Malaga (13%) and Almería (5%) (Perez-Perez *et al.*, 2005) constituting 95% of national production**.** Furthermore, currently 9348 Has. of land in the Municipality of Murcia has been planted with lemon trees, which

implies an amounts of 41% of the arable land of the Murcia province (Acosta *et al.*, 2011). In recent years, the production of lemon fruits in Murcia Region is the most important among *Citrus* fruits and all ligneous crops, only approaching them the peach production.

(3.2.) LEMON BIOACTIVE COMPOUNDS

Lemon fruit contains many important natural chemical components, including phenolic compounds (mainly flavanones) and other nutrients and non-nutrients (vitamins, minerals, dietary fiber, essential oils and carotenoids). The health-promoting effects and properties of lemons have been associated with their contents, namely vitamin C and flavonoids, due to their natural antioxidant characteristics (Proteggente *et al.*, 2002; Vinson *et al.*, 2001).

More than sixty individual flavonoids have been identified in *Citrus* species and most of them can be classified into three groups: flavanones, flavones and flavonols (Abad-García *et al.*, 2012; Mellisho *et al.*, 2011). In addition, other phenolic compounds (phenolic acids, etc.) are also present in these *Citrus* species.

3.2.1. Flavonones

Hesperidin (Hesperitin 7-*O*-rutinoside) and eriocitrin (Eriodictyol 7-*O*-rutinoside) are the main flavanones in lemon juice (Caristi *et al.*, 2003; González-Molina *et al.*, 2008b; Y. Miyake *et al.*, 2006), although two isomers of hesperidin, neohesperidin (Hesperitin 7-*O*-neohesperoside) and homoeriodictyol 7-*O*-rutinoside, have also been identified (A. Gil-Izquierdo *et al.*, 2004). On the other hand, the peel is rich in neohesperidin, neoeriocitrin (Eriodictyol 7-*O*-neohesperoside) and naringin (Naringenin 7-*O*-neohesperoside), being also detected minor amounts of narirutin (Naringenin 7-*O*-rutinoside) (Bocco *et al.*, 1998) (Kawaii *et al.*, 1999) (Table 3.2).

Although lemon flavonoid concentration depends on madurity, variety, etc. (Vandercook & Tisserat, 1989), the peel is richer than lemon juice (Tripoli *et al.*, 2007). Recently, eriodictyol 7-*O*-rutinoside 4´-*O*-glucoside and isosakuranetin-7-*O*-rutinoside heve been detected in low concentrations and only in some cultivars (Abad-García *et al.*, 2014).

Table 3.2. Structure of main lemon flavanones.

Flavanones	R_7	$R_{3'}$	$R_{4'}$
Hesperitin 7-*O*-rut	*O*-rut	OH	OCH_3
Eriodictyol 7-*O*-rut	*O*-rut	OH	OH
Homoeriodictyol 7-*O*-rut	*O*-rut	OCH_3	OH
Naringenin 7-*O*-neohesp	*O*-neohesp	H	OH
Hesperitin 7-O-neohesp	*O*-neohesp	OH	OCH_3
Eriodictyol 7-O-neohesp	*O*-neohesp	OH	OH
Naringenin 7-O-rut	*O*-rut	H	OH
Eriodictyol 7-*O*-rut 4′-*O*-glc	*O*-rut	OH	*O*-glc

rut: rutinoside, neohesp: neohesperidoside. Substituents are associated to the figure 2.2.

3.2.2. Flavones

In lemon fruit, the two mainly *C*-glucosylflavones are diosmetin 6,8-di-*C*-glucoside and diosmetin 6-*C*-D-glucoside (Y. Miyake *et al.*, 1997), particularly abundant also in lime, but almost absent in other *Citrus* fruits (Yoshiaki Miyake *et al.*, 1998). Lower amounts of vicenin-2 (Apigenin 6,8-di-*C*-glucoside), and diosmin (Diosmetin 7-*O*-rutinoside) have been also identified in lemon juices (Benavente-García & Castillo, 2008; A. Gil-Izquierdo *et al.*, 2004; González-Molina *et al.*, 2008b). In addition, chrysoeriol 6,8-di-*C*-glucoside, apingenin 7 (malonylapiosyl)-glucoside (A. Gil-Izquierdo *et al.*, 2004; Mellisho *et al.*, 2011), and diosmetin 8-*C*-D-glucoside have been detected (Benavente-García *et al.*, 2008) (Table 3.3).

Table 3.3. Structure of main lemon flavones.

Flavones	R_6	R_7	R_8	$R_{3'}$	$R_{4'}$
Diosmetin 6,8-di-*C*-glc	Glc	OH	Glc	OH	OCH_3
Diosmetin 7-*O*-rut	H	*O*-rut	H	OH	OCH_3
Apigenin 6,8-di-*C*-glc	Glc	OH	Glc	H	OH
Chrysoeriol 6,8-di-*C*-glc	Glc	OH	Glc	OCH_3	OH
Luteolin 6,8-di-*C*-glc	Glc	OH	Glc	OH	OH
Luteolin 7-*O*-rut	H	*O*-rut	H	OH	OH
Apigenin 7-(mal)-glc	H	*O*-Ap-glc	H	H	OH
Diosmetin 6-*C*-D-glc	Glc	OH	H	OH	OCH_3
Diosmetin 8-*C*-D-glc	H	OH	Glc	OH	OCH_3
Luteolin	H	OH	H	OH	OH

rut: rutinoside, glc:glucoside, mal: malonylapiosyl. R substituents are associated to the figure 2.3.

Other flavones have been recently described in lemon juice in trace contents (Abad-García *et al.*, 2014).

3.2.3. Flavonols

Rutin (Quercetin 3-*O*-rutinoside), and myricetin are the most abundant flavonols identified in lemon juice (Dugo *et al.*, 2005; Hertog *et al.*, 1993), while quercetin and kaempferol are in both peel and juice (Kawaii *et al.*, 1999; Wang *et al.*, 2008; Wang *et al.*, 2007). Quercetin 3-*O*-rutinoside-7-*O*-glucoside has been also found (A. Gil-Izquierdo *et al.*, 2004). Iso/limocitrol 3-β-glucoside, limocitrin 3-β-d-glucoside and limocitrol were detected, as polymethoxylated flavonols, in peel (Dugo *et al.*, 2005) (Table 3.4).

Table 3.4. Structure of main lemon flavonols.

Flavonols	**R_3**	**R_6**	**R_8**	**$R_{3'}$**	**$R_{4'}$**	**$R_{5'}$**
Quercetin 3-*O*-rut	*O*-rut	H	H	OH	OH	OCH_3
Quercetin	OH	H	H	OH	OH	OCH_3
Kaempferol	OH	H	H	H	OH	OH
Myricetin	OH	H	H	OH	OH	OH
Iso-limocitrol 3-β-glc	*O*-glc	OCH_3	OCH_3	OH	OCH_3	OH
Limocitrin 3-β-glc	*O*-glc	OCH_3	OCH_3	OH	OH	OH
Limocitrol	OH	OCH_3	OCH_3	OCH_3	OH	OH

rut: rutinoside, glc:glucoside. R substituents are associated to the figure 2.4.

Quercetin 7-*O*-rutinoside, isorhamnetin-3-*O*-rutinoside-7-*O*-glucoside, isorhamnetin-7-*O*-rutinoside and isorhamnetin-3-*O*-rutinoside have also been recently quantified in lemon juices (Abad-García *et al.*, 2014).

3.2.4. Phenolic acids

In lemon fruits, hydroxycinnamic acids (caffeic, ferulic, sinapic and *p*-coumaric acids, chlorogenic acid (3-*O*-caffeoylquinic acid), and neochlorogenic acid (5-*O*-caffeoylquinic acid)) (García-Salas *et al.*, 2013; González-Molina *et al.*, 2008a; Mellisho *et al.*, 2011; Y. C. Wang *et al.*, 2008), in addition to benzoic acids (protocatechuic, *p*-hydroxybenzoic and vanillic acids) have been detected (Y. C. Wang *et al.*, 2007; Xu *et al.*, 2008) (Table 3.5). Furthermore, 1-feruloyl-β-D-glucopyranoside and 1-sinapoyl-β-D-glucopyranoside was identified by Miyake et al. (Y. Miyake *et al.*, 2007) identified in lemon juice.

Table 3.5. Structure of main lemon phenolic acids

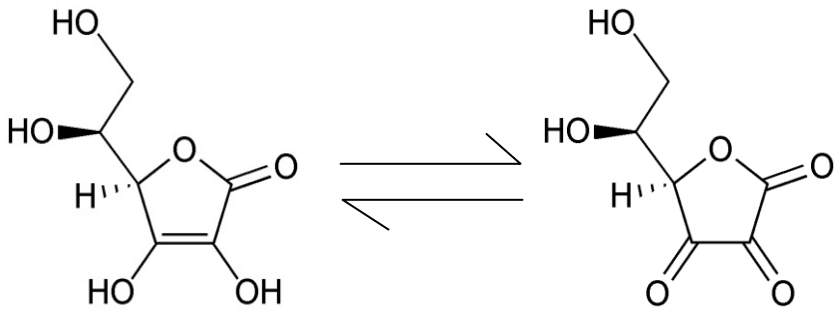
Hydroxycinnamic acids (A)	**R_1**	**R_2**	**R_3**	**R_4**
Caffeic acid	H	H	OH	OH
Ferulic acid	H	H	OH	OCH_3
Sinapic acid	H	OCH_3	OH	OCH_3
p-cumaric acid	H	H	OH	H
Benzoic acids (B)				
Protocatechuic acid	H	OH	OH	-
p-hydroxybenzoic acid	H	OH	H	-
Vanillic acid	H	OH	OCH_3	-

R substituents are associated to the figure 2.7.

3.2.5. Other nutrients in lemon

It is well established that lemon fruit and its by-products constitute an interesting source not only of phenolic compounds but also for nutrients and non-nutrient (vitamins, minerals, dietary fiber, essential oils, organic acids and carotenoids), which are essential for the normal growth and the correct functioning of the human physiological systems (W. C. Kim *et al.*, 2004).

Vitamin C has a very important nutritional role, and the symptoms of its absence in the diet have been known since the time of the ancient Egyptians and Greeks (Martí *et al.*, 2009). It is one the most important water-soluble vitamin involved in the cell physiology and crucial processes of human health, as well as an efficient scavenger of reactive oxygen species (Figure 3.4) (González-Molina *et al.*, 2010; S. K. Lee & Kader, 2000). Vitamin C can be identified as ascorbic acid (AA), although with a reversible reaction of oxidation-reduction results in its oxidized form, dehydroascorbic acid (DHAA), which also has vitamin bioactivity (Figure 3.4) (Halliwell, 1996). For this reason, vitamin C must be considered as the sum of AA and DHAA. Nevertheless, AA and DHAA can suffer an irreversible degradation to hydroxymethyl furfural mainly due to temperature and oxygen, being consequence in juices of non-enzymatic browning, and losing their activity (Martí *et al.*, 2009).



AA DHAA

Figure 3.4. General structure of vitamin C: Ascorbic acid (AA) and dehydroascorbic acid (DHAA).

Vitamin C is usually considered the major antioxidant in *Citrus* fruits and juices. Lemon juice is a major source of vitamin C in human diet (40 mg/100 mL approximately), similar to orange juice (Angel Gil-Izquierdo *et al.*, 2002; González-Molina *et al.*, 2008b), being a 45-60% of the current US Recommended Dietary Allowance (RDA) in healthy adults (between 65 and 90 mg, depends on gender) (EFSA, 2010).

Lemon is also a rich source of micronutrients. Other vitamins present in minor quantities are A and B group (B1, B2, B3, B6 and B9). Besides, the major mineral present in lemon is potassium (K), although other minerals like calcium (Ca), magnesium (Mg) and phosphorus (P) are also present in minor levels 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).

About the essential oils in lemon, about sixty individual components (Russo *et al.*, 1998) have been described. The major component is D-limonene (45–75%). On the other hand, citric acid is the most representative organic acid in lemon (Kefford & Chandler, 1970), comprising as much as 8% in dry weight, that represents 5–6 g/100 mL (Penniston *et al.*, 2008).

Lemon is a rich source of carotenoids, and mature lemons accumulates β-cryptoxanthin predominantly in the flavedo and juice sacs (Kato *et al.*, 2004).

(3.3.) LEMON AND HEALTH

The role of lemon in folk medicine is widely accepted, and in recent years the scientific literature is prolific in references to the beneficial health effects from the *Citrus* consumption in general and lemon in particular. The beneficial effects of the consumption of *Citrus* fruits can be attributed, not only to the vitamin C, minerals, dietary fiber, essential oils, organic acids and carotenoids, but also to the bioactivity of their flavonoids. Overall, lemon fruits, rich in flavonoids, are a very important part of a balanced diet, particularly for their role in prevention of diseases, such as obesity, diabetes, blood lipid lowering, cardiovascular diseases, and certain types of cancer (Adibelli *et al.*, 2009; Benavente-García *et al.*, 2008; González-Molina *et al.*, 2010; Y. Miyake *et al.*, 2006).

3.3.1. Lemon and oxidative stress

There is widespread evidence that many diseases are related to the imbalance between antioxidant defenses and reactive oxygen species or free radicals, such as cancer, cardiovascular disorders, diabetes, renal disease, diverse dementia, and the biological process of aging, which increase as a function of the magnitude of oxidative stress (Packer *et al.*, 2008). Thus, it is important to maintain this oxidative balance to avoid health alterations and it is in this point where lemon phytochemicals can act enhancing the effectiveness of the antioxidant defense system.

Is well known that Vitamin C is a very important and powerful antioxidant that works in aqueous environments of the body. Its primary antioxidant partners are Vitamin E and the carotenoids, as well as working along with the antioxidant enzymes. Vitamin C co-operates with Vitamin E to regenerate α-tocopherol from α-tocopherol radicals in membranes and lipoproteins (Kojo, 2004). Vitamin C also reduces redox active transition metal ions in the active sites of specific biosynthetic enzymes (Carr & Frei, 1999). Nonetheless, the intake of very high doses of Vitamin C, suggested initially by Linus Pauling, has been a subject of intense debate for many years (Cameron & Pauling, 1976). While intake of high doses of Vitamin C (up to 2000 mg/day) has not been consistently reported to result in side effects, the benefit of these intakes of Vitamin C has never been established, suggesting that in these high amounts may act as pro-oxidant (Carr *et*

al., 1999). However, recommended intakes are between 60 and 90 mg (Food and Nutrition Board, 2013) and lemon juice contains 40mg/100mL approximately, being essential in order to keep the oxidative balance of the body.

Lemon flavonoids have been also suggested as antioxidants (Masuoka *et al.*, 2012; Yuting *et al.*, 1990) and it has been confirmed on biological systems. Regarding to lemon flavanones, eriocitrin has been reported to be a potent antioxidant and to have suppressive effect on oxidative stress in rats (Minato *et al.*, 2003). Hesperidin has also been shown to decrease the oxidative stress in rat liver and kidney (Tirkey *et al.*, 2005) and to have inhibitory activity on non-enzymatic lipid peroxidation in the rat brain mitochondria (Ratty & Das, 1988). Other *Citrus* flavonoids have been reported as antioxidants in animal models, like rutin (Kamalakkannan & Prince, 2006) or diosmetin (Villa *et al.*, 1992).

A point worth mentioning is how lemon phytochemicals really act against oxidative stress. Original flavonoids are usually absorbed and metabolized in other bioavailable metabolites that result from digestive or hepatic activity and may differ from the native substances in terms of biological activity (Claudine Manach *et al.*, 2004). Therefore, these *in vivo* metabolites, and not the original phenolics present in fruits, seem to be the real responsible for the protective effects linked to their consumption, and not necessarily because a radical scavenging ability. Moreover, 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).

3.3.2. Lemon and cardiovascular diseases

The beneficial effects of flavonoid consumption on cardiovascular risk are supported by mechanistic and epidemiologic evidence (Hooper *et al.*, 2008). Regarding to *Citrus* flavonoids, El-Shafae *et al.* (El-Shafae & El-Domiaty, 2001), reported that hesperidin and diosmin could be effective for the treatment of chronic venous insufficiency, chronic hemorrhoids and venous leg ulcer. Moreover, polymethoxylated flavones from *Citrus* peel were recently reported to have anti-cardiovascular disease function (Green *et al.*, 2013). Treatment with

naringenin in mice for 6 months, led to a marked reduction in the progression of atherosclerosis (Mulvihill *et al.*, 2010), and in rabbits fed a high-cholesterol diet, supplementation of 0.1% naringin or 0.05% naringenin for 8 weeks decreased aortic fatty streak area when compared to controls (C. H. Lee *et al.*, 2001). The ability of hesperitin to modulate apoB secretion and cellular cholesterol homeostasis has also been determined in human hepatoma cell line (Wilcox *et al.*, 2001). Flavones have a considerable anti-thrombotic activity and provoke a reduction of ischemic damage, interfering with the activity of macrophagic NOS (Nijveldt *et al.*, 2001).

There are clinical studies about the effect of lemon juice on blood pressure in treatment of hypertension (Adibelli *et al.*, 2009). Vicenin-2 and diosmetin 6,8-di-*C*-glucoside from lemon juice showed a suppressive effect on the expression of blood adhesion molecules (Y. Miyake *et al.*, 2007). In this sense, other recently study reported that after six months of citrus-based juice consuming, there are significant variations in cardiovascular risk factors in Metabolic Syndrome patients (Mulero *et al.*, 2012).

Finally, lemon phytochemicals can improve some body disorders related to cardiovascular health, such as diabetes (Aruoma *et al.*, 2012; Bahorun *et al.*, 2012), obesity (Dallas *et al.*, 2008; Ferrara, 2007) or inflammation (Galati *et al.*, 1994). Moreover both eriocitrin and hesperetin metabolites played an important role in plasma, serum and hepatic lipids, with lipid-lowering activities in vivo in high-cholesterol fed rats (H. K. Kim *et al.*, 2003; Y. Miyake *et al.*, 2006).

3.3.3. Lemon and cancer

Dietary flavonoids have been considered to be chemo-preventive or anticancer agents (Hertog *et al.*, 1993). *Citrus* flavonoids exert their anticancer effects through three mainly mechanisms: Protection against DNA damage, inhibition of tumor development and inhibition of cell proliferation (Manthey *et al.*, 2001).

Flavonoids can protect DNA by their ability to absorb ultraviolet light. Some experiments on a UV irradiated model of plasmidic DNA indicate a protecting effect of naringenin and rutin against UV-induced damage of DNA (Kootstra, 1994). Indeed, naringin plays an important role in regulating

antioxidative capacity by increasing superoxide dismutase and catalase activities and by up-regulating the gene expressions of superoxide dismutase, catalase, and glutathione peroxidase in cholesterol-rich diet-fed rabbits (Jeon *et al.*, 2001).

Flavonoids can also act by affecting tumor promotion at the beginning of carcinogenesis by an increase of the detoxification processes. In particular, *Citrus* flavonoids inhibit ornithine decarboxylase induction of skin tumor promotion, increasing the inorganic phosphate ^{32}P incorporation rate in the phospholipid membrane and activation of protein kinase C (C. Manach *et al.*, 1996). Hesperetin and naringenin were tested for their abilities to inhibit human breast cancer cell proliferation *in vitro* (So *et al.*, 1996). Miller *et al.* (Miller *et al.*, 2008) studied the inhibition of oral carcinogenesis by *Citrus* flavonoids in hamsters and the antineoplastic activity, concluding that hesperetin, neohesperetin, tangeretin and nobiletin were ineffective, while naringin and naringenin gave good results. Synthetic hesperidin and diosmin were effective as chemopreventive agents in urinary-bladder carcinogenesis (Yang *et al.*, 1997).

Citrus flavonoids can inhibit invasion, by rat malignant cells, in cardiac and hepatic tissue of syngenetic rats (Bracke *et al.*, 1989). Hydroxycinnamates, glycosylated flavonoids and the polymethoxylated flavones have shown inhibitory activity on several tumoral cell line proliferations (Manthey *et al.*, 2001). Other studies showed eriocitrin and its aglycone, eriodictyol, as potent inhibitors of lipoxygenases, which are involved in the biosynthesis of various bioregulators that are closely related to the pathogenesis of several diseases such as allergy and atherosclerosis and cancer (Nogata *et al.*, 2007). Hesperidin in different *Citrus* juices also showed antiproliferative activity (Camarda *et al.*, 2007), reporting lemon in particular potent antiproliferative activities on HepG2 human liver-cancer cell growth in a dose-dependent manner (Sun *et al.*, 2002).

Also the positive effect of Vitamin C in reducing the incidence of stomach cancer have been studied, being most probably due to the inhibitory action in the generation of nitrous compounds by interrupting the reaction between nitrites and amine groups (You *et al.*, 2000), although it has recently shown that this effect may be due to a cytotoxic effect of vitamin C on human gastric cancer cell line AGS (Nagappan *et al.*, 2012). Consistent protective effect of Vitamin C has also been found in lung and colorectal cancer (Kojo, 2004).

3.3.4. Lemon and neurological disorders

Unlike cancer or cardiovascular diseases, protective effects of lemon on neurological disorders have been scarcely studied. One *in vitro* study reported that hesperidin, naringenin and their metabolites are able to enter the brain endothelium and cross the blood-brain barrier (Youdim *et al.*, 2003). Although some recently data also suggests that hesperidin exerts its neuroprotective effect against rotenone due to its antioxidant, maintenance of mitochondrial function, and antiapoptotic properties in a neuroblastoma cell line (Tamilselvam *et al.*, 2013), Hwang *et al.*, (Hwang & Yen, 2009) demonstrate that hesperidin, isorhamnetin and isosakuranetin are involved in neuroprotection against oxidative stress acting more as signaling molecules than antioxidants. Hesperidin also protected cortical neurons from oxidative injury by activating prosurvival Akt and ERK1/2 signaling pathways (Vauzour *et al.*, 2007).

Other lemon flavonoid, rutin, has also demonstrated neuroprotective effects in rats, attributable to its inhibitory effect against microglial activation and its role in synapse formation via neurotrophic factors in the hippocampus (Koda *et al.*, 2009). Likewise, lemon flavone apigenin also may inhibits the production of NO and PGE_2 in microglia and inhibits neuronal cell death in a middle cerebral artery occlusion-induced focal ischemia mice model (Ha *et al.*, 2008).

3.3.5. Lemon and other diseases

The most significant benefits against diseases are the aforementioned, however, there are other therapeutic properties of lemon juice that should pointed out. Thus, juice is very rich in citric acid which is efficient to prevent metabolic pathologies (Ferrara, 2007). Knowledge of citric acid content of beverages may be useful in nutrition therapy for calcium urolithiasis, especially for patients with hypocitraturia. The intake of beverages containing citric acid, like lemon juice, increase the total volume of urine, reducing the saturation of calcium and other crystals, and may enhance urinary citrate excretion, since citrate is a naturally occurring inhibitor of urinary crystallization (Penniston *et al.*, 2008).

Lemon juice also showed significant antimicrobial activity (De Castillo *et al.*, 2000). Hesperidin has been reported to possess therapeutic value for the clinical

treatment of rheumatoid arthritis in rats (Li *et al.*, 2008), and this flavanone and naringenin also inhibit some enzymes resulting in drug interactions to obtain increased oral bioavailability of drugs (Gattuso *et al.*, 2007).

It is mentionable that the essential oils can be used as antimicrobial and antifungal agents (Sharma & Tripathi, 2006). Moreover, these oils, mainly citral, also reported antiviral activity which constitutes an interesting alternative to the antiviral drugs because of their weaker toxicity (Minami *et al.*, 2003), and others showed certain hepatoprotective activity (Bhavsar *et al.*, 2007). On the other hand, both kinds of lemon fiber (soluble pectins and insoluble), exhibited several beneficial effects on human health, including delay in gastric emptying, that reduces the energy absorption, prevents of a surge in blood glucose levels and interferes with the reabsorption of bile acids that reduce plasma cholesterol levels (Blackburn *et al.*, 1984); prevention of colorectal cancer (Goodlad, 2001); and stimulation of the intestinal cell proliferation (Fukunaga *et al.*, 2003).

4. BERRY FRUITS

Berry fruits, small fruits or berries, are fleshy fruits, generally refered to any small fruit that lacks seeds and can be eaten whole (Figure 4.1), and nowadays many of them are understudied, even though they possess numerous beneficial effects (Basu *et al.*, 2010; Del Rio *et al.*, 2010; Shukitt-Hale *et al.*, 2008).

Figure 4.1. Common berry fruits.

Strawberry and blackberry are two common examples. Berry fruits are popularly consumed not only in fresh and frozen forms but also as processed and derived products including canned fruits, yogurts, beverages, and jams and jellies. In addition, there has been a growing trend in the intake of berry extracts as ingredients in functional foods and dietary supplements, which may or may not be combined with other colorful fruits, vegetables, and herbal extracts (Seeram, 2008).

Berries are low in calories and are high in moisture and fiber. They contain natural antioxidants such as vitamins C and E, and micronutrients such as folic acid, calcium, selenium, alpha and beta carotene, and lutein (Basu *et al.*, 2010). Phytochemicals found in berries include (poly)phenolics along with high proportions of flavonoids, highlighting anthocyanins. Other phenolic compounds found are flavonols, procyanidins, ellagitannins and phenolic acids, among others (Del Rio *et al.*, 2010).

Berries are widely distributed and including the widest known blackberry (*Rubus* spp.), black raspberry (*Rubus occidentalis*), blueberry (*Vaccinium corymbosum*), cranberry (*Vaccinium oxycoccus*), red raspberry (*Rubus idaeus*), chokeberry (*Aronia* spp.) and strawberry (*Fragaria* spp.). Other understudied berries but with great for potential health benefits are maqui (*Aristotelia chilensis* (Molina) Stuntz), açaí (*Euterpe oleraceae* Mart.) and sloe (*Prunus spinosa* L.), studied in this Doctoral Thesis.

(4.1.) MAQUI BERRY

Aristotelia chilensis (Molina) Stuntz, commonly referred to as "Maqui Berry", is a member of the *Elaeocarpaceae* family, native to the temperate rain forests in Southern Chile as well as part of neighboring Argentina. The plant is both medicinal and edible and can be found at all altitudes. It generally grows near streams or in valleys, and prefers slightly acidic, humid environments, moderately fertile and well-drained soils. The tree stands up to 5 meters in height, but generally appears as a bush or shrub (Stuntz, 1914). *Aristotelia chilensis* yields a small edible purple/black berry averaging 5 mm in diameter with typically 3−4 seeds (Schreckinger, Wang, *et al.*, 2010) (Figure 4.2).

Figure 4.2. *Aristotelia chilensis* (Mol.) Stuntz (Maqui): Tree and berry.

4.1.1. Production, varieties and commercial products

Taking into account that it is a wild fruit, its industrial production is developing from Latin America. According to the Chilean authorities, the estimated area of Maqui from Coquimbo Region in the North of the country to the Aysen Region in the South is 170,000 Has, including the islands of Juan Fernandez and Chiloe. Nonetheless, the real potential of harvesting the fruit of maqui is much lower than this since most of the total estimated is very difficult to access. The time of collection of the fruit occurs from December to March each year and depends directly on the geographical distribution, being the earliest collection while further north is the tree. In relation to the productivity, it is known that a plant on average at age 7 can produce up to 10 kg of fruit, which would be directly related to the quality (Fondef Project, 2012). On the other hand, no different cultivars or varieties of maqui have been found. Fruits are usually eaten fresh or used for juice and jams (Figure 4.3).

Figure 4.3. Examples of maqui berry products in the market.

4.1.2. Maqui bioactive compounds: Anthocyanins

Maqui berry contains numerous essential nutrients: vitamin B, minerals, omega-3, 6, as well as 9 fatty acids, and also proteins. Moreover, maqui berry powder and other products are also a good source of dietary fiber. The maqui berries also contain a considerable percentage of vitamin C and trace elements, highlighting the presence of Br, Zn, Cl, Co, Cr, Vn, Tn, and Mo. However, therapeutic properties of maqui have been related to their high quantity of phenolic compounds, such as flavonols, ellagic acid derivatives, and phenolic acids, but specially anthocyanins (Schreckinger, Wang, *et al.*, 2010), presenting maqui eight different glycosides and diglycosides of cyanidin and delphinidin (Céspedes, Valdez-Morales, *et al.*, 2010; Escribano-Bailón *et al.*, 2006; Fredes *et al.*, 2014) (Table 4.1).

Table 4.1. Chemical structure of anthocyanins found in maqui berry

Anthocyanins	R_3	R_5	$R_{3'}$	$R_{5'}$
Delphinidin 3-samb 5-glc	Samb	OH	OH	OH
Delphinidin 3,5-diglc	Glc	Glc	OH	OH
Delphinidin 3-samb	Samb	OH	OH	OH
Delphinidin 3-glc	Glc	OH	OH	OH
Cyanidin 3-samb 5-glc	Samb	Glc	OH	H
Cyanidin 3,5 diglc	Glc	Glc	OH	H
Cyanidin 3-samb	Samb	OH	OH	H
Cyanidin 3-glc	Glc	OH	OH	H

Glc: glucoside, Samb: sambubioside (hexoside+pentoside). R substituents are associated to the figure 2.5.

4.1.3. Other bioactive compounds of maqui

Moreover, other flavonoids have been recently described in maqui, such as flavonols (quercetin and myricetin derivatives), phenolic acids (5-O-caffeoylquinic, *p*-coumaric, sinapic, benzoic, and gallic acid), catechins and

proanthocyanidins (Céspedes, Valdez-Morales, *et al.*, 2010), but in much lower amounts compared to anthocyanins contents, being these coloured flavonoids the main representatives of the *Aristotelia chilensis* phytochemicals.

4.1.4. Maqui and health

In the traditional native herbal medicine, infusions of maqui fruits and leaves have long been used to treat sore throats, kidney pain, digestive diseases (tumors and ulcers), fever, and scarring injuries (Suwalsky *et al.*, 2008). This fruit has also been recently reported as one of the healthiest berries, due to these bioactive components, but the studies on their health benefits are not too extended, probably because is a not worldwide known fruit.

Maqui berry have demonstrated to possess high antioxidant capacity (Rubilar *et al.*, 2011; Schreckinger, Wang, *et al.*, 2010), and *in vitro* inhibition of adipogenesis and inflammation (Céspedes, Alarcon, *et al.*, 2010; Schreckinger, Wang, *et al.*, 2010). Moreover, maqui berry extracts suppress the light-induced photoreceptor cell death by inhibiting ROS production (Tanaka *et al.*, 2013). One study have demonstrated that juice and phenolic extracts inhibit LDL oxidation and protect human endothelial cells against oxidative stress (Miranda-Rottmann *et al.*, 2002), showing other research an alteration of human erythrocyte morphology of flavonoids from maqui berry (Suwalsky *et al.*, 2008).

In vitro and *in vivo* antidiabetic effects have also been reported (Rojo *et al.*, 2011; Rubilar *et al.*, 2011), showing cardioprotective effects on acute ischemia/reperfusion performed in rat heart *in vivo* (Céspedes *et al.*, 2008). One recent study suggested that maqui berry induces changes in the aggregation kinetics of Aβ producing variations in the nucleation phase, and altering Thioflavin T insertion in β-sheets; which demontrated important neuroprotective effects (Fuentealba *et al.*, 2012).

(4.2.) AÇAÍ BERRY

Euterpe oleracea Mart (Figure 4.4) is a berry from a palm tree, which is native from of the Southern America: Caribbean, Mesoamerica, Northern South America, and Western South America. Nonetheless, it is in the region of the

Amazon River estuary which is the largest and densest natural populations of this palm tree with an area estimated of 10000-25000 km^2 (Calzavara, 1972).

Figure 4.4. *Euterpe oleraceae* Mart (Açaí): Tree and berry

The fruit, commonly known as açaí berry, is a small, round, black-purple drupe about 25 mm in circumference, similar in appearance to a grape, but smaller and with less pulp and produced in branched panicles of 500 to 900 fruits (Marcason, 2009).

4.2.1. Production, varieties and commercial products

Açaí palm can be found both wild and cultivated. Because it is a cross pollinating specie it has a wide variation of types for different traits of interest such as earliness, fruit yield, pulp yield and production time. The main world producer is the state of Pará in Brazil, being responsible for the 85% of the world production, although under current conditions of production and marketing, obtaining accurate data is almost impossible due to the lack of control in sales as well as the lack of a streamlined production. Between 1996 and 2002 the production area of açaí palm in Brazil changed from 9223 to 18816 hectares, 92.1% corresponding to the State of Pará. The annual production in Brazil is around 160000 tons of açaí berries and the 20% is for local consumption (Nogueira, 2006).

The various types of açaí were defined according to the fruit color, forms clumps and clusters, number of fruits per bunch and diameter of stems. From these characteristics resulted different "ethnovarieties", calling açaí-purple or black, white açaí (remain green in their mature stage), açaí-assu, açaí-sword and açaí-ox-blood. Black and purple açaí are considered the most common (Nogueira,

2006) (Sanabria & Sangronis, 2007).

The juice and pulp of açaí fruits are used in various juice blends, smoothies, sodas, and other beverages. The frozen pulp, jam and juice are used to flavour ice cream and other frozen treats, cakes, porridges and bonbons (Muñoz *et al.*, 2014). Açaí pulp has become a trend in southern Brazil where it is consumed fresh. Processed powder from the fruit pulp is beginning to be sold as a health food in loose powder or capsule form. The powder can also be used as natural food coloring additive.

4.2.2. Açaí bioactive compounds

Açaí pulp has a high nutritional value: lipids can account for up to 50%, proteins for about 10% of the dry matter and calories up to 247 calories/100 g (Muñoz *et al.*, 2014). Açaí juice is also rich in calcium, fiber and minerals like zinc, magnesium and potassium, vitamin E and in antioxidants, mainly flavonoids, and specially anthocyanins.

Anthocyanins, proanthocyanidins, and other flavonoids were found to be the major phytochemicals in freeze-dried açaí fruit pulp/skin powder. Two anthocyanins, cyandin 3-glucoside and cyanidin 3-rutinoside, were found to be predominant; and peonidin 3-rutinoside, peonidin 3-glucoside and pelargonidin 3-glucoside were also found as minor anthocyanins (Table 4.2) (Del Pozo-Insfran *et al.*, 2004; Lichtenthäler *et al.*, 2005; Schauss *et al.*, 2006).

Table 4.2. Chemical structure of anthocyanins found in açaí berry

Anthocyanins	**R_3**	**R_5**	**$R_{3'}$**	**$R_{5'}$**
Cyanidin 3-glc	Glc	OH	OH	H
Cyanidin 3-rut	Rut	OH	OH	H
Peonidin 3-glc	Glc	OH	OCH_3	H
Peonidin 3-rut	Rut	OH	OCH_3	H
Pelargonidin 3-glc	Glc	OH	H	H

Glc: glucoside, Rut rutionoside. R substituents are associated to the figure 2.5.

Cyanidin 3-sambubioside, cyanidin 3-arabinboside, peonidin 3-(6''-malonylglucoside), and delphinidin 3-(6''-malonylglucoside) have been detected in trace amounts (Gordon *et al.*, 2012; Hogan *et al.*, 2010; Jensen *et al.*, 2008), as well as other phenolic compounds: quercetin and kaempferol glycosides, phenolic acids, homoorientin, orientin, isovitexin, scoparin, and chrysoeriol among others (Mulabagal & Calderón, 2012).

4.2.3. Açaí and health

Açaí berry is commonly known as "*The magic fruit of Amazon or the fruit of life*." This has led to a high number of scientific publications about their close relationship with health benefits. In this sense, the antioxidant capacities of all purple açaí samples were found to be excellent against peroxyl radicals, good against peroxynitrite and poor against hydroxyl radicals compared with common European fruit and vegetable juices recently analyzed (Kang *et al.*, 2010; Lichtenthäler *et al.*, 2005).

In vitro and *in vivo* antioxidant and anti-inflammatory activities were also found in the fruit and in a berry juice blend (Jensen *et al.*, 2008). Through these antioxidant and anti-inflammatory activities, açaí juice attenuates atherosclerosis in ApoE deficient mice (Xie *et al.*, 2011). Other *in vivo* results demonstrated that that açaí polyphenols prevent endothelial dysfunction and vascular structural changes in renovascular hypertensive rats (da Costa *et al.*, 2012).

The intake of açaí fruit inhibited mouse urinary bladder carcinogenesis, also probably due to its potential antioxidant action (Fragoso *et al.*, 2012). Antiproliferative effects have also been found in anthocyanin rich extract from açaí, in CaCo-2 intestinal cells, and in rat brain glioma cells (Hogan *et al.*, 2010; Pacheco-Palencia *et al.*, 2010). Likewise, one open label clinical pilot study reported pain reduction and improvement in range of motion after daily consumption of an açaí pulp-fortified polyphenolic-rich fruit and berry juice blend (Jensen *et al.*, 2011).

Açaí seed extract protected mice with high-fat diet from phenotypic and metabolic characteristics of metabolic syndrome (de Oliveira *et al.*, 2010), and showed reduced levels of selected markers of metabolic disease risk in overweight adults eating açaí pulp (Udani *et al.*, 2011). The hypocholesterolemic

activity of açaí has been also recently demonstrated (De Souza *et al.*, 2012).

Recent studies suggested also neuroprotective effects of açaí pulp and its bioactives: on mouse brain BV-2 microglial cells (Poulose *et al.*, 2012), showing that cyanidin 3-glucoside (characteristic anthocyanin inaçaí) neuroprotective effects in mice with focal cerebral ischemia (Min *et al.*, 2011).

(4.3.) SLOE BERRY

Prunus spinosa L. (sloe, blackthorn, or bair) is a species of *Prunus* native to Europe, western Asia, and locally in northwest Africa (Rushforth, 1999). It is also locally naturalised in New Zealand and eastern North America. *Prunus spinosa* is a large deciduous shrub or small tree growing to 5 metres tall, with blackish bark and dense, stiff, spiny branches. The fruit, called a "sloe", is a drupe 10–12 millimetres in diameter, black with a purple-blue waxy bloom, ripening in autumn, and are thin-fleshed, with a very strongly astringent flavour when fresh (Rushforth, 1999) (Figure 4.5).

Figure 4.5. *Prunus spinosa* L. (Sloe): Tree and berry.

4.3.1. Production, varieties and commercial products

Sloe production is limited only to small industries of liquors or jams elaboration, so is not possible to estimate the world production. Is important to note that sloe is also used in the elaboration of alcoholic drinks in Spain, France, Germany, United Kingdom, and other central European countries, standing out the Spanish "Pacharán" (Figure 4.6).

Figure 4.6. Traditional "Pacharán" elaborated with sloe berries

This "digestive" liquor is a traditional alcoholic beverage obtained by maceration (6 months) of sloe berries in an aqueous ethanol liqueur (25% alcohol by volume, approximately) that contains sugar and essential oils of aniseed (*Pimpinella anisum* L. or *Illicium verum* H.) (Fernández-García *et al.*, 1998). Nowadays, industrial production is located in northern Spain, primarily Navarra, where it has been a typical and traditional digestive drink since the 1400s (Barros *et al.*, 2010). The liquor has an intense and attractive red color, owing to the anthocyanin contribution of the sloe berries during maceration (Ganhão *et al.*, 2010).

4.3.2. Sloe bioactive compounds

Sloe berries have an important nutritional value, containing carbohydrates, proteins and fats as macronutrients. Fruits also have good values of vitamin C, vitamin E, and vitamin A, representing a good source of these healthy vitamins. Furthermore other vitamins of B complex and minerals are also present in minor quantities (Sikora *et al.*, 2013).

It is known that sloe berries present four major anthocyanins (cyanidin-

glucoside, cyanidin-rutinoside, peonidin-glucoside, and peonidin-rutinoside), flavonol derivatives, and hydroxycinnamic acid derivatives; in accordance with previous researches (Barros *et al.*, 2010; Deineka *et al.*, 2005). Recently, other anthocyanins have been detected in trace amounts: cyanidin and peonidin pentosides, and cyanidin and peonidin acetylglucosides (Guimarães *et al.*, 2013). The four mainly anthocyanins found in sloe berry are the same described before for açaí berry (Figure 3.7).

4.3.3. Sloe and health

Sloe phytochemical composition is directly correlated with their health benefits, although characterization of its potential for health related benefits remain quite understudied, being the research about health benefits of this fruit rather limited.

Sloe berry is an excellent astringent. It can be used in treatment of diarrhea. Its pectin components have a soothing and relaxing effect on stomach inflammations. Sloe berries can be used for stimulation of our metabolism, and can be of very good use in cases of eczema, herpes, allergies, colds, catarrh, indigestion, kidney stones, and skin and bladder disorders (Ganhão *et al.*, 2010). Sloe fruit is also cited as diuretic and purgative (Barros *et al.*, 2010), and have recently been proved as antioxidant (Fraternale, Giamperi, Bucchini, & Ricci, 2009; Sikora *et al.*, 2013).

CHAPTER 2. OBJECTIVES

The growing interest and needs of tasty, convenient and affordable added-value foods and drinks with health-promoting properties is triggering the development of beverages, juices and other drinks enriched with fruits, as source of nutrients and bioactive compounds.

The main objective of this Doctoral Thesis was to design new functional beverages with attractive organoleptic attributes and health added-value, rich in bioactive compounds, based on lemon juice with added Spanish and Latin American berries. In order to achieve this main objective, the following specific objectives were also conducted:

- To compare different Latin American and Spanish fruits with respect to their "antidiabesity" (α-glucosidase and lipase inhibition) and antioxidant capacities.
- The design novel, safe and acceptable drinks, blends of lemon juice with, maqui, açaí, sloe, or chokeberry, in order to determine future applications in nutrition and cognitive health, analyzing their phytochemical content and their anticholinergic capacity.
- To determine how storage conditions may affect the phytochemicals, color and bioactivities of a selected new beverage (made of lemon juice and maqui-berries at different concentrations)
- The design of a new isotonic beverages, enriched with polyphenols from lemon juice and berries, which may be useful to support sport nutrition and active healthy living.
- To elaborate an aniseed liquor-based beverage with maqui, following the traditional elaboration of "Pacharán", comparing between liquors in order to establish the potential consumer preference for this leisure beverage or liquor.

CHAPTER 3. PUBLICATIONS SELECTED FOR THE PRESENT DOCTORAL THESIS

1. PUBLICATIONS 1 AND 2: EVALUATION OF PHYTOCHEMISTRY AND BIOACTIVITY OF DIFFERENT LATIN-AMERICAN AND SPANISH CITRUS FRUITS

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Evaluation of Latin-American fruits rich in phytochemicals with biological effects

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ABSTRACT

This work aimed to provide a thorough description of the polyphenolic composition of five Latin-American fruits of increasing interest, which have certain anti-diabetic effects (açaí, maqui, Cape gooseberry, papaya and noni), and to correlate their antioxidant capacity and anti-diabesity activities (lipase and α-glucosidase inhibition), and examine their potential use by the food industry. The phytochemical profiling of the fruits revealed a wide range of bioactive phenolics. The inhibition of pancreatic lipase was significant for maqui, and maqui and papaya were the best inhibitors of α-glucosidase. Regarding the DPPH, $ABTS^{\cdot+}$ and FRAP assays, maqui berries displayed the highest activity. The ORAC method and the superoxide radical scavenging assays revealed maqui and açaí as the best performers. These Latin-American fruits are of great value regarding nutrition and health benefits, and the development of products for the control of diabetes and obesity.

1. Introduction

In the last decade, numerous publications have dealt with the high content of bioactive compounds, mostly polyphenols, present in certain fruits and their protective effects on human health. Besides their antioxidant properties, it is widely accepted that most of these phenolics have an affinity for proteins, exhibiting inhibitory activity on some functional enzymes (Birari & Bhutani, 2007). In this aspect, the inhibition of α-glucosidase, a key enzyme that catalyzes the final step in the digestive process of carbohydrates, could delay the breakdown of oligosaccharides and disaccharides into monosaccharides, diminishing glucose absorption and consequently reducing postprandial hyperglycaemia (Rubilar et al., 2011). Berry polyphenols have been reported as being inhibitors of α-glucosidase *in vitro* (Boath, Stewart, & McDougall, 2012). The current therapeutic approaches for the treatment of obesity involve the inhibition of dietary triacylglycerol absorption, via inhibition of pancreatic lipase (PL) by orlistat (Birari & Bhutani, 2007). Many polyphenolic extracts are active against this enzyme; for example, polyphenol-rich water extracts from litchi (*Litchi chinensis* Sonn.) show *in vitro* inhibitory effects (Wu et al., 2013), while extracts from certain berries have been described as effective inhibitors of PL *in vivo* (McDougall, Kulkarni, & Stewart, 2009).

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The increase in human obesity has been accompanied by a growing incidence of diabetes. The close relationship between these two conditions has led to the adoption of the term *diabesity* (Schröder, 2007). In this sense, some fruits of Latin-American origin represent good sources of bioactive compounds with certain anti-diabetic effects and are receiving increasing interest. Açaí (*Euterpe oleracea* L.) is a palm-tree berry from the Amazon area in South America. Potential benefits have been attributed to açaí fruits, extracts and juices: antioxidant, anti-inflammatory (Schauss et al., 2006), hypocholesterolaemic (De Souza et al., 2012) and anti-diabetic activity (Kim, Hong, Jung, Jeong, & Cho, 2012). Maqui (*Aristotelia chilensis* L.) is a common edible berry from central and southern Chile, and it is a source of natural colorant due to the presence of anthocyanins. Various reports have linked the phenolics of maqui berries with their high antioxidant capacity (Rubilar et al., 2011), *in vitro* inhibition of adipogenesis and inflammation (Schreckinger, Wang, Yousef, Lila, & De Mejia, 2010), cardioprotection (Céspedes, El-Hafidi, Pavon, & Alarcon, 2008) and *in vitro* and *in vivo* anti-diabetic effects (Rojo et al., 2011; Rubilar et al., 2011). Cape gooseberry (*Physalis peruviana* L.) is an herbaceous perennial semi-shrub that grows in sub-tropical zones. Its calyx represents an essential source of carbohydrates during the first 20 days of growth and development. Anti-inflamatory, hypocholesteroleamic and antihepatotoxic effects have been attributed to *P. peruviana* (Ramadan, 2012). Papaya (*Carica papaya* L.) fruits grow in tropical and sub-tropical regions and are marketed around the world. Numerous papers have described beneficial effects of this fruit against chronic diseases such as cancer (Nguyen, Shaw, Parat, & Hewavitharana, 2013), diabetes (Juárez-Rojop et al., 2012) and obesity (Athesh, Karthiga, & Brindha, 2012). Noni (*Morinda citrifolia* L.) is a tropical and sub-tropical plant used as a folk medicine in Pacific islands to treat a broad range of diseases. Recently, several health benefits have been attributed to noni fruits, juice or extracts, namely hypolipidemic and anti-oxidative effects (Lin et al., 2012), hepatoprotection (Wang, Nowicki, Anderson, Jensen, & West, 2008), anti-diabetic (Sabitha, Adhikari Prabha, Shetty Rukmini, Anupama, & Asha, 2009) and anti-cancer (Brown, 2012) effects.

In addition to the above-mentioned bioactivities, it has been reported that these five fruits also display significant anti-diabetic activity (Juárez-Rojop et al., 2012; Kim et al., 2012; Lee et al., 2012; Pujiyanto, Lestari, Suwanto, Budiarti, & Darusman, 2012; Rojo et al., 2011). To the best of our knowledge, there are insufficient data in the literature (arising from the same assaying procedure and conditions) to allow a comprehensive comparison of the different antioxidants and enzymatic activities of these polyphenol-rich fruits. Moreover, the polyphenolic composition has been reported for some of these fruits, mainly açaí and maqui, but phenolic characterization studies on Cape gooseberry, noni and papaya are scarce. Hence, the aim of this study was to evaluate the α-glucosidase- and lipase-inhibitory activities and the antioxidant activities of five fruits rich in bioactive compounds (native from different countries in Latin America), together with their phytochemical profiling, making a comparison of the species, their origin and the analytical methods studied.

2. Material and methods

2.1. Chemicals

The compounds 2,2-diphenyl-1-picrylhidracyl radical (DPPH·), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)diammonium salt (ABTS·+), 2,4,6-tripyridyl-S-triazine (TPTZ), ferric chloride hexahydrate, fluorescein (free acid), 2,2′-azobis(2-methylpropionamidine) dihydrochloride (APPH), monobasic sodium phosphate, dibasic sodium phosphate, Folin Ciocalteu's Reagent, β-nicotinamide adenine dinucleotide (NADH), phenazine methosulphate (PMS), nitrotetrazolium blue chloride (NBT), triazine hydrochloride, 4-nitrophenyl α-D-glucopyranoside, α-glucosidase from *Saccharomyces cerevisiae*, acarbose and potassium phosphate were obtained from Sigma–Aldrich (Steinheim, Germany). Meanwhile, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and magnesium chloride hexahydrate were purchased from Fluka Chemika (Neu-Ulm, Switzerland); sodium carbonate (anhydrous), sodium benzoate, and potassium sorbate were bought from Panreac Química S.A. (Barcelona, Spain). LIPASE-PS™ (Kit) was obtained from Trinity Biotech (Jamestown, NY, USA). Ultrapure water was produced using a Millipore water purification system.

2.2. Fruits

Lyophilized maqui^{CHI2}, açaí^{BRZ2}, noni^{ECU} and papaya^{ECU} fruits, provided by Ecuadorian Rainforest LLC. (Belleville, NJ, USA), were obtained from Chile (CHI), Brazil (BRZ) and Ecuador (ECU). Açaí^{COL} was supplied by Corpocampo S.A. (Bogotá, Colombia (COL)). Açaí^{BRZ1} was provided by Amazon Dreams Industria e Comercio S.A. (Belem, Pará, Brazil). Cape gooseberry^{COL} fruits and calyx were provided by Arc. Eurobanan S.L. (Santa Fé de Bogotá, Colombia). Maqui^{CHI1} and maqui^{CHI3} were provided by INTA-UCHILE (Santiago, Chile): maqui^{CHI1} was lyophilized and maqui^{CHI3} was spray-dried and microencapsulated by atomization.

2.3. Extraction

Each sample (100 mg) was mixed with 1 mL of methanol/water (70:30, v/v). For the HPLC analysis samples were acidified with 1% of formic acid. Then, the samples were vortexed and sonicated in an ultrasonic bath for 60 min. The samples were kept at 4 °C overnight and sonicated again for 60 min. A centrifugation (model EBA 21, Hettich Zentrifugen) step (9500 *xg*, 5 min) was used to separate the supernatant from the solid residue. This supernatant was filtered through a 0.45-μm PVDF filter (Millex HV13, Millipore, Bedford, MA, USA) and stored at 4 °C before the analyses were performed.

2.4. Identification of phenolic compounds by HPLC–DAD–ESI/MS^n and quantification by RP-HPLC–DAD

The chromatographic analyses for the identification were carried out on a Luna C18 column (250 × 4.6 mm, 5 mm particle size; Phenomenex, Macclesfield, UK). Water/formic acid (99:1, v/v) and acetonitrile were used as the mobile phases A

and B, respectively, with a flow rate of 1 mL/min. The linear gradient started with 8% solvent B, reaching 15% solvent B at 25 min, 22% at 55 min, and 40% at 60 min, which was maintained to 70 min. The injection volume was 30 μL. Chromatograms were recorded at 280, 320, and 360 nm. The HPLC–DAD–ESI/MS*n* analyses were carried out using an Agilent HPLC 1100 series model equipped with a photodiode array detector and a mass detector in series (Agilent Technologies, Waldbronn, Germany). The equipment consisted of a binary pump (model G1312A), an autosampler (model G1313A), a degasser (model G1322A), and a photodiode array detector (model G1315B). The HPLC system was controlled by ChemStation software (Agilent, version 08.03). The mass detector was an ion trap spectrometer (model G2445A) equipped with an electrospray ionization interface, and was controlled by LCMSD software (Agilent, version 4.1). The ionization conditions were 350 °C and 4 kV, for capillary temperature and voltage, respectively. The nebulizer pressure and nitrogen flow rate were 65.0 psi and 11 L/min, respectively. The full-scan mass covered the range of *m*/*z* from 100 to 1200. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas, with voltage ramping cycles from 0.3 to 2 V. The mass spectrometry data were acquired in the positive ionization mode for anthocyanins and in the negative ionization mode for other flavonoids. The MS*n* was carried out in the automatic mode on the more-abundant fragment ion in MS($n-1$).

For the quantification, all samples were also centrifuged for 5 min at 9500*g*. Each supernatant was filtered through a 0.45-μm PVDF filter (Millex HV13, Millipore, Bedford, MA, USA) before injection into the HPLC system, as described by Gironés-Vilaplana, Villaño, Moreno, and García-Viguera (2013). Chromatograms were recorded at 280, 320, 360 and 520 nm. Anthocyanins were quantified as cyanidin 3-*O*-glucoside at 520 nm, flavonols and xanthone derivatives as quercetin 3-*O*-glucoside at 360 nm, ellagic acid derivatives as ellagic acid 3-*O*-glucoside at 360 nm and cinnamic acids as 5-*O*-caffeoylquinic acid at 320 nm.

2.5. α-Glucosidase inhibitory activity

The α-glucosidase inhibitory activity was assessed by modification of a previously-reported procedure (Chan, Sun, Reddy, & Wu, 2010). Briefly, each well contained 100 μL of 2 mM 4-nitrophenyl α-D-glucopyranoside in 10 mM potassium phosphate buffer (pH 7.0) and 20 μl of the sample or acarbose (positive control), also in buffer. The reaction was initiated by the addition of 100 μL of the enzyme solution (56.66 mU/mL). The plates were incubated at 37 °C for 10 min. The absorbance of the 4-nitrophenol released from 4-nitrophenyl α-D-glucopyranoside was measured at 400 nm. The increase in absorbance was compared with that of the control (buffer instead of sample solution), to calculate the inhibitory activity and the IC_{50} (sample concentration which reduced the enzyme concentration by 50%).

2.6. Lipase inhibitory effect

Lipase-PS™ reagents were obtained from Trinity Biotech (Procedure No. 805, Trinity Biotech, Jamestown, NY, USA). The lipase activity was determined in microscale 96-well micro plates (Nunc, Roskilde, Denmark) in an Infinite® M200 micro plate reader (Tecan, Grödig, Austria), as described by Gironés-Vilaplana et al. (2013). The recorded rate of increase in absorbance at 550 nm, due to the formation of quinone diimine dye, was used to determine the pancreatic lipase activity in the samples prepared.

2.7. Antioxidant capacity

The free radical scavenging activities were determined using the DPPH·, $ABTS^{\cdot+}$, and FRAP (ferric reducing antioxidant power) methods adapted to a microscale, according to Mena et al. (2011). The antioxidant activity was evaluated by measuring the variation in absorbance at 515 nm after 50 min of reaction with the radical (for DPPH·), at 414 nm after 50 min ($ABTS^{\cdot+}$), and at 593 nm after 40 min for FRAP. The assays were performed using 96-well micro plates (Nunc, Roskilde, Denmark) and an Infinite® M200 micro plate reader (Tecan, Grödig, Austria). All the reactions were started by adding 2 μL of the corresponding diluted sample to the well containing the stock solution (250 μL). The final volume of the assay was 252 μL. The antioxidant activity was also determined using the ORAC-FL assay, according to Ou, Hampsch-Woodill, and Prior (2001). The results were expressed as mM Trolox/100 mg dry weight.

The superoxide radical ($O_2^{\cdot-}$) scavenging activity was also determined spectrophotometrically, in a 96-well plate reader, by monitoring the effect of extracts on the $O_2^{\cdot-}$ induced reduction of NBT at 560 nm. Superoxide radicals were generated by the NADH/PMS system, according to a described procedure (Ferreres et al., 2009). The experiments were performed in triplicate and the results expressed as the IC_{50}.

2.8. Statistical analysis

The data are presented are mean values ($n = 3$) ± standard deviation. All the data were subjected to analysis of variance (ANOVA) and a Multiple Range Test (Tukey's test), using IBM SPSS statistics 21 software (SPSS Inc., Chicago, IL, USA). Pearson's correlation analysis was performed to corroborate the relationships between selected parameters.

3. Results and discussion

3.1. Phenolic compounds

The HPLC–DAD–ESI/MS^n analysis of the hydromethanolic extracts of the Latin-American fruits revealed a wide range of different phenolic compounds. Anthocyanins and ellagic acid derivatives were only detected in açai and maqui fruits (Table 1), whilst flavonols, xanthones and hydroxycinnamic acid derivatives were widely distributed in the fruits (Table 2).

The açaí fruit samples (açaí^{BRZ1}, açaí^{BRZ2} and açaí^{COL}) contained diverse anthocyanins (cyanidin-*O*-hexosides and pelargonidin and peonidin rutinosides (**A7**, **A8**, **A10**, **A12** and **A13**)) and flavonols (quercetin, kaempferol and isorhamnetin glycosides (**F6**, **F8**, **F13**, **F14**, **F16**, **F17**, **F18**, **F19**)), in accordance with other reports (Gironés-Vilaplana, Valentão, et al., 2012;

Table 1 – Anthocyanins and ellagic acid derivatives identified and quantified (mg/100 g dried weight) in açaí and maqui fruits.

Compounds	Rt	$[M-H]^+$	MS^n	Fruits					
				Açaí^{BRZ1}	Açaí^{BRZ2}	Açaí^{COL}	Maqui^{CHI1}	Maqui^{CHI2}	Maqui^{CHI3}
Anthocyanins									
A1 Delphinidin 3-O-sambubioside-5-O-glucoside	5.8	759	465, 303	–	–	–	250.25 ± 11.44	125.21 ± 24.65	354.51 ± 27.49
A2 Delphinidin 3,5-O-diglucoside	6.6	627	465, 303	–	–	–	240.35 ± 8.45	251.45 ± 19.31	114.38 ± 1.34
A3 Cyanidin 3,5-O-diglucoside	11.7	611	449, 287	–	–	–	134.65 ± 3.28*	77.07 ± 3.60*	11.47 ± 0.90*
A4 Cyanidin 3-O-sambubioside-5-O-glucoside	12.0	743	581, 287	–	–	–			
A5 Delphinidin 3-O-sambubioside	15.7	597	303	–	–	–	63.22 ± 0.37	18.63 ± 1.87	72.45 ± 6.22
A6 Delphinidin 3-O-glucoside	16.5	465	303	–	–	–	210.90 ± 1.84	110.69 ± 3.41	167.55 ± 12.58
A7 Cyanidin 3-O-galactoside	17.7	449	287	55.60 ± 7.81	10.49 ± 0.22	27.21 ± 1.92	–	–	–
A8 Cyanidin 3-O-glucoside	19.6	449	287	–	3.89 ± 0.36	–	–	–	–
A9 Cyanidin 3-O-sambubioside	20.2	581	287	–	–	–	82.21 ± 0.48	23.44 ± 2.71	56.29 ± 4.61
A10 Cyanidin-3-O-rutinoside	21.6	595	287	81.49 ± 9.49	31.21 ± 1.62	305.21 ± 30.01	–	–	–
A11 Cyanidin 3-O-glucoside-5-O-rhamnoside	22.4	595	449, 287	–	–	–	2.54 ± 1.87	7.59 ± 1.87	1.96 ± 0.75
A12 Pelargonidin 3-rutinoside	25.2	579	433, 271	28.88 ± 1.48	–	1.60 ± 0.32	–	–	–
A13 Peonidin 3-O-rutinoside	26.8	609	463, 301	3.45 ± 0.97	3.44 ± 0.52	13.78 ± 6.90	–	–	–
Total				143.42 ± 16.71	49.02 ± 1.82	347.81 ± 35.86	984.12 ± 7.32	614.08 ± 47.44	881.84 ± 46.07
Ellagic acid derivatives									
EA1 Granatin B	26.4	951	933, 301	–	–	–	0.53 ± 0.11	0.54 ± 0.13	0.39 ± 0.00
EA2 Ellagic acid hexoside	34.3	463	301	–	–	–	2.01 ± 0.15	1.23 ± 0.00	1.17 ± 0.08
EA3 Dehydrogaloyl-hexahydroxydiphenoyl hexoside	40.1	615	463, 301	–	–	–	14.29 ± 0.20	12.42 ± 1.54	5.88 ± 0.38
Total				–	–	–	16.83 ± 0.42	14.20 ± 1.48	7.44 ± 0.43

* **A3** and **A4** coeluted and were quantified together in maqui^{CHI1}, maqui^{CHI2} and maqui^{CHI3}. Rt: retention time of.

Table 2 – Non-red polyphenols and hydroxycinnamic acids identified and quantified (mg/100 g dried weight) in Latin-American fruits: açaí, maqui, Cape gooseberry, papaya, and noni.

Non-red polyphenols					Açaí	Maqui				Cape goseberry		Papaya	Noni
	Rt	$[M-H]^-$	MS^n	BRZ1	BRZ2	COL	CHI1	CHI2	CHI3	Fruit	Calyx	ECU	ECU
F1	19.8	771	609, 301	–	–	–	–	–	–	–	34.56 ± 1.39	–	–
F2	25.1	755	593, 285	–	–	–	–	–	–	–	5.00 ± 0.13	–	–
F3	25.6	421	366, 241	–	–	–	–	–	–	–	–	0.84 ± 0.13	–
F4	27.0	631	479, 317	–	–	–	3.20 ± 0.24	1.95 ± 0.31	1.71 ± 0.17	–	–	–	–
F5	30.3	479	317	–	–	–	2.47 ± 0.34	2.01 ± 0.30	2.07 ± 0.18	–	–	–	–
F6	32.5	447	285	8.05 ± 0.67	–	5.18 ± 0.50	–	–	–	–	–	–	–
F7	32.8	479	317	–	–	–	1.92 ± 0.38	2.58 ± 0.58	2.46 ± 0.18	–	–	–	–
F8	33.4	447	285	9.98 ± 0.71	–	7.96 ± 1.04	–	–	–	–	–	–	–
F9	33.8	573	366, 241	–	–	–	–	–	–	–	–	4.90 ± 1.12	–
F10	34.7	269	-	–	–	–	–	–	–	–	–	–	11.37 ± 1.03
F11	35.3	573	366, 241	–	–	–	–	–	–	–	–	0.78 ± 0.20	–
F12	37.2	741	609, 301	–	–	–	–	–	–	–	–	–	0.86 ± 0.37
F13	40.6	609	301	3.53 ± 0.32		6.17 ± 0.86	5.13 ± 0.87	7.18 ± 1.65	2.46 ± 0.58	1.74 ± 0.54	147.57 ± 2.55	0.72 ± 0.24	4.04 ± 1.14
F14	42.5	463	301	–	5.34 ± 1.12	–	2.17 ± 0.60	1.28 ± 0.49	2.42 ± 0.71	–	–	1.30 ± 0.56	–
F15	44.0	463	301	–	–	–	–	1.70 ± 0.70	–	–	–	1.65 ± 0.21	–
F16	47.8	433	301	–	1.46 ± 0.21	–	1.55 ± 0.09	3.71 ± 0.60	1.16 ± 0.19	–	–	–	–
F17	50.2	433	301	–	2.76 ± 0.03	–	2.24 ± 0.09	3.74 ± 0.29	2.74 ± 0.58	–	–	–	–
F18	51.9	593	285	1.10 ± 0.10	–	1.57 ± 0.08	0.74 ± 0.29	0.53 ± 0.17	0.55 ± 0.06	0.44 ± 0.17	8.31 ± 0.01	–	2.47 ± 0.05
F19	54.4	623	315	1.42 ± 0.33	–	0.68 ± 0.11	–	–	–	–	–	–	–
F20	55.3	447	301	–	0.82 ± 0.27	–	–	–	–	–	–	–	–
			TOTAL	24.07 ± 3.37	17.75 ± 0.91	21.56 ± 2.18	19.42 ± 2.40	24.41 ± 1.47	15.58 ± 1.82	2.18 ± 0.71	195.44 ± 3.88	10.20 ± 2.08	18.74 ± 2.82
Hydroxycinnamic acids													
Q1	7.9	515	191	–	6.68 ± 0.17	–	–	–	–	–	–	–	–
Q2	11.6	353	191, 179	–	18.84 ± 0.61	–	–	–	–	–	71.54 ± 2.62	–	–
Q3	14.8	431	-	–	–	–	–	–	–	–	–	–	1.06 ± 0.14
Q4	18.9	353	191	–	–	–	–	8.84 ± 0.86	–	–	51.72 ± 2.48	–	–
Q5	30.1	337	191	–	7.33 ± 0.77	–	–	–	–	–	–	–	–
			TOTAL	–	32.86 ± 1.17	–	–	8.84 ± 0.86	–	–	123.26 ± 5.10	–	1.06 ± 0.14

Quantified at 360 nm: **F1**: Quercetin 3-O-rutinoside 7-O-hexoside, **F2**: Kaempferol 3-O-rutinoside 7-O-hexoside, **F3**: Mangiferin, **F4**: Myricetin 3-O-galoylglucoside, **F5**: Myricetin 3-O-galactoside, **F6**: Kaempferol 3-O-galactoside, **F7**: Myricetin 3-O-glucoside, **F8**: Kaempferol 3-O-glucoside, **F9**: Mangiferin gallate, **F10**: Lucidin, **F11**: Isomangiferin gallate, **F12**: Quercetin 3-O-rutinoside-7-O-pentoside, **F13**: Quercetin 3-O-rutinoside, **F14**: Quercetin 3-O-galactoside, **F15**: Quercetin 3-O-glucoside, **F16**: Quercetin 3-O-xyloside, **F17**: Quercetin 3-O-arabinoside, **F18**: Kaempferol 3-O-rutinoside, **F19**: Isorhamnetin 3-O-rutinoside, **F20**: Quercetin 3-O-rhamnoside. Quantified at 320 nm: **Q1**: 3,5-O-Dicaffeoylquinic acid, **Q2**: 3-O-Caffeoylquinic acid, **Q3**: Asperulosidic acid, **Q4**: 5-O-Caffeoylquinic acid, **Q5**: 5-O-p-Coumaroylquinic acid. Samples were labelled as follows: açaí[1]: açaí^{BRZ1}, açaí[2]: açaí^{BRZ2}, açaí[3]: açaí^{COL}, maqui[1]: maqui^{CHI1}, maqui[2]: maqui^{CHI2}, maqui[3]: maqui^{CHI3}.

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Table 3 – α-glucosidase inhibition and pancreatic lipase activity (U/L) of all the fruits.

Fruit		α-Glucosidase IC_{50}^{*}	Lipase U/L
Açaí			
BRZ1		–	120.91 ± 10.95 ef
BRZ2		2.14 ± 0.18 a	51.03 ± 12.70 bc
COL		–	137.46 ± 16.27 f
Maqui			
CHI1		0.33 ± 0.02 a	19.62 ± 3.39 a
CHI2		1.10 ± 0.17 a	26.82 ± 5.48 ab
CHI3		0.81 ± 0.08 a	21.30 ± 2.92 a
Cape gooseberry			
Fruit (COL)		56.03 ± 0.32 c	180.83 ± 14.98 g
Calyx (COL)		–	100.22 ± 9.57 de
Papaya			
ECU		1.58 ± 0.26 a	107.58 ± 11.16 e
Noni			
ECU		27.32 ± 2.79 b	74.63 ± 4.37 cd
LSD	$P < 0.05$	0.988	8.388

Means ($n = 3$) in the same columns followed by different letters are significantly different at $P < 0.05$ according to Tukey's test.
* Samples without data did not inhibit 50% of enzyme.

Gordon et al., 2012). Considering the origin of the fruits, only açaí^{BRZ2} had hydroxycinnamic acid derivatives (3,5-*O*-dicaffeoylquinic (**Q1**), 3-*O*-caffeoylquinic (**Q2**) and 5-*O*-*p*-coumaroylquinic acids (**Q5**)), but displayed significantly-smaller amounts of anthocyanins (49.02 ± 1.82 mg/100 g dry weight (dw) total anthocyanins content (TAC)) compared to açaí^{BRZ1} (TAC: 143.42 ± 16.71 mg/100 g dw) and açaí^{COL} (TAC: 347.81 ± 35.86 mg/100 g dw). Cyanidin 3-*O*-rutinoside (**A10**) was the major anthocyanin quantified in açaí fruits (56.8, 63.7, and 87.7% of the total anthocyanins in açaí^{BRZ1}, açaí^{BRZ2} and açaí^{COL}, respectively), followed by cyanidin 3-*O*-galactoside (**A7**) (Table 1). Regarding flavonols, the levels were similar among the three studied açaís, but the phytochemical profile of açaí^{BRZ2} was completely different from those of açaí^{BRZ1} or açaí^{COL} (Table 2). This was probably due to differences in the ripening stage or growth conditions, which are directly related to the nutritional profile of these fruits (Gordon et al., 2012).

With respect to the maqui berry powders, different glycosides and di-glycosides of delphinidin and cyanidin were found (**A1**, **A2**, **A3**, **A4**, **A5**, **A6**, **A9**, **A11**) (Table 1), in accordance with previous reports (Gironés-Vilaplana, Mena, García-Viguera, & Moreno, 2012; Gironés-Vilaplana, Valentão, et al., 2012). Flavonols (quercetin and myricetin derivatives (**F4**, **F5**, **F7**, **F13**, **F14**, **F15**, **F16**, **F17**, **F18**)), one ellagic acid hexoside (**EA2**), and two ellagitannins (granatin B (**EA1**) and dehydrogaloyl-hexahydroxydiphenoyl hexoside (**EA3**), the latter identified for the first time in maqui fruits) were also identified at quantifiable levels (Table 1). It is important to note the higher anthocyanin concentrations in maqui with respect to açaí, particularly in maqui^{CHI1} (TAC: 984.12 ± 7.32 mg/100 g dw). Moreover, maqui^{CHI2} exhibited a slightly-higher level of flavonols than the other two maquis, but also a lower content of anthocyanins (Table 2). Furthermore, maqui^{CHI2} was the only maqui fruit containing 5-*O*-caffeoylquinic acid (**Q4**), while maqui^{CHI3} had the lowest level of flavonols.

The Cape gooseberry fruit is protected by an accrescent calyx; to the best of our knowledge, there are no reports on its phenolic content. For this reason, the calyx and fruit were analyzed separately; this enabled us to show that there were more compounds and a much-higher level of phenolics in the calyx^{COL} (195.44 ± 3.88 mg/100 g dw) than in the fruit^{COL} (2.18 ± 0.71 mg/100 g dw). Two different quercetin (**F1**, **F13**) and kaempferol glycosides (**F2**, **F18**) were identified and quantified in the calyx, but only trace amounts of quercetin 3-*O*-rutinoside (**F13**) and kaempferol 3-*O*-rutinoside (**F18**) were found in the fruit. The 3-*O*-caffeoylquinic (**Q2**) and 5-*O*-caffeoylquinic (**Q4**) acids were also identified in the calyx, but not in the fruit. A point worth mentioning is that the Cape gooseberry calyx^{COL} had the highest concentrations of flavonols and hydroxycinnamic acid derivatives among all the Latin-American fruits analyzed.

In papaya^{ECU} powder, mangiferin (**F3**) and the galloylated forms of mangiferin (**F9**) and isomangiferin (**F11**) (Table 2), xanthone glycosides, were identified. These were described as potent antidiabetic agents by Sellamuthu, Muniappan, Perumal, and Kandasamy (2009). Mangiferin gallate (**F9**) was the major flavonoid present in papaya^{ECU} (4.90 ± 1.12 mg/100 g dw). Quercetin 3-*O*-rutinoside (**F13**), previously reported for this fruit (Andarwulan et al., 2012; Rivera-Pastrana, Yahia, & González-Aguilar, 2010), was also found, as were both quercetin hexosides (**F14**, **F15**) – at lower levels. Hydroxycinnamic acid derivatives were absent or below the detection threshold.

The noni^{ECU} fruit contained low levels of some flavonol glycosides: quercetin 3-*O*-rutinoside-7-*O*-pentoside (**F12**), quercetin 3-*O*-rutinoside (**F13**), and kaempferol 3-*O*-rutinoside (**F18**) (Table 2). Previous work identified the rutinosides of quercetin and kaempferol (Andarwulan et al., 2012; Dussossoy et al., 2011), but not quercetin 3-*O*-rutinoside-7-*O*-pentoside (**F12**), identified and quantified for the first time in this work. Moreover, lucidin (**F10**) (an anthraquinone characteristic of noni (Deng, West, Jensen, Basar, & Westendorf,

2009)) was also detected and quantified in significant amounts (11.37 ± 1.03 mg/g dw), as well as asperulosidic acid (**Q4**), previously reported by Dussossoy et al. (2011).

3.2. α-glucosidase inhibition

The enzyme α-glucosidase catalyzes the final step in the digestion and breakdown of carbohydrates, so its inhibition can be effective for the regulation of Type II diabetes, by controlling glucose absorption (Rubilar et al., 2011). The IC_{50} values were calculated in order to compare the different Latin-American fruits, as shown in Table 3: açaí^{BRZ1}, açaí^{COL} and Cape gooseberry calyx^{COL} did not reach 50% inhibition of the enzyme activity, while Cape gooseberry fruit^{COL} and noni^{ECU} caused slight inhibition and açaí^{BRZ2}, papaya^{ECU}, maqui^{CHI1}, maqui^{CHI2} and maqui^{CHI3} were exceptionally effective, with lower IC_{50} values than the acarbose positive control (IC_{50} = 3.89 ± 0.79). The *in vitro* anti-diabetic effect of maqui has been previously reported (Rubilar et al., 2011), as have the *in vitro* and *in vivo* effects of its anthocyanins (Rojo et al., 2011). Moreover, myricetin and delphinidin, present in maqui berries, have been reported as the best α-glucosidase inhibitors among the flavonoids (Tadera, Minami, Takamatsu, & Matsuoka, 2006). Papaya fruit was also particularly effective against α-glucosidase, probably due to mangiferin (**F3**) and its derivatives (**F9**, **F11**), which are associated with a strong anti-diabetic effect (Sellamuthu et al., 2009). Several studies have also reported that some parts of the *Carica papaya* plant, such as the leaves, exert significant hypoglycaemic effects (Juárez-Rojop et al., 2012). As described above, açaí^{BRZ2} possessed lower amounts of phenolic compounds than other açaís but exhibited a different flavonoid profile, especially with regard to flavonols (Tables 1 and 2), which probably explains its higher activity with respect to açaí^{BRZ1} and açaí^{COL}. The α-glucosidase inhibition results are consistent with the idea that the differences among fruits in their phytochemical profiles, and the interactions between compounds in the fruit matrix, can contribute to their distinct activities; myricetin, quercetin, delphinidin and mangiferin derivatives being the most relevant (Sellamuthu et al., 2009; Tadera et al., 2006). To support this, the α-glucosidase results were correlated with the TAC ($R^2 = -0.527^{*}$, $P < 0.05$) and with the total non-red polyphenols ($R^2 = -0.652^{**}$, $P < 0.01$). Previous research showed that adding crowberry – containing different types of polyphenols – to blackcurrant juice improved the postprandial glycaemic control in healthy subjects (Törrönen et al., 2012). In this sense, maqui, papaya, and açaí fruits may offer dietary coadjuvants to control hyperglycaemia in diabetic patients; however, further evaluation of their *in vivo* anti-diabetic activity is necessary to verify these beneficial effects.

3.3. Pancreatic lipase inhibition

The inhibition of pancreatic lipase, which splits triacylglycerols into absorbable monoacylglycerol and fatty acids, is the main prescribed treatment for obesity in developed countries (McDougall et al., 2009). In order to find alternative sources for obesity prevention and treatment, we searched for inhibitory action of the Latin-American fruits on lipase activity. The results are given in Table 3, expressed in U/L (lipase activity). The activity of the lipase standard was 254 U/L. The maqui fruits exhibited the lowest values and, therefore, the greatest inhibitory effect on pancreatic lipase (26.82, 19.62 and 21.30 U/L for maqui^{CHI1}, maqui^{CHI2} and maqui^{CHI3}, respectively). This result is in line with the strong effect of maqui on lipid metabolism demonstrated previously, specifically the ability of maqui phenolic extracts to reduce adipogenesis and lipid accumulation in 3T3-L1 adipocytes (Schreckinger et al., 2010). The lipase inhibition was correlated strongly with the TAC ($R^2 = -0.682^{***}$, $P < 0.001$), maqui fruits exhibiting the highest anthocyanin content among the fruits examined. A result similar to the

Table 4 – Antioxidant activity of all the fruits.

Fruit	DPPH· mmol Trolox/100 g	ORAC mmol Trolox/100 g	$ABTS^{+}$ mmol Trolox/100 g	FRAP mmol Trolox/100 g	$O_2^{\cdot -}$ IC_{50} (mg/mL)
Açaí					
BRZ1	7.06 ± 0.13 d	28.30 ± 3.88 ef	21.10 ± 1.41 cd	15.99 ± 0.55 c	0.69 ± 0.03 ab
BRZ2	4.80 ± 0.09 b	12.21 ± 0.36 bc	9.17 ± 0.45 b	7.23 ± 0.89 a	0.80 ± 0.11 ab
COL	6.38 ± 0.15 cd	30.36 ± 1.45 ef	20.87 ± 0.75 c	15.79 ± 0.87 c	0.75 ± 0.05 ab
Maqui					
CHI1	13.93 ± 0.65 f	29.90 ± 0.98 ef	25.48 ± 0.82 d	25.42 ± 2.64 d	0.67 ± 0.02 a
CHI2	10.81 ± 1.37 e	26.80 ± 1.64 ef	22.12 ± 3.71 cd	18.12 ± 2.36 c	0.76 ± 0.10 ab
CHI3	6.76 ± 0.25 d	18.18 ± 0.29 d	18.30 ± 1.91 c	16.13 ± 0.96 c	1.83 ± 0.11 d
Cape gooseberry					
Fruit (COL)	1.60 ± 0.06 a	3.29 ± 0.58 a	3.11 ± 0.61 a	4.92 ± 1.24 a	13.91 ± 0.52 e
Calyx (COL)	4.94 ± 0.05 bc	24.29 ± 3.11 e	8.58 ± 1.26 b	11.42 ± 1.59 b	1.19 ± 0.08 bc
Papaya					
ECU	4.41 ± 0.28 b	8.71 ± 0.32 ab	7.09 ± 0.76 ab	6.39 ± 0.66 a	36.43 ± 0.30 f
Noni					
ECU	3.71 ± 0.17 b	15.08 ± 0.22 cd	9.04 ± 0.99 b	6.92 ± 0.85 a	1.37 ± 0.08 cd
LSD $P < 0.05$	0.409	1.707	1.274	1.171	0.155

Means ($n = 3$) in the same columns followed by different letters are significantly different at $P < 0.05$ according to Tukey's test.

α-glucosidase inhibition was also found in açaí fruits regarding lipase inhibition, açaí^{BRZ2} being very active, in contrast to the other two açaís, owing to its differing phytochemical profile. Furthermore, the α-glucosidase and lipase results were directly correlated ($R^2 = 0.828^{***}$, $P < 0.001$). Certain hypocholesterolaemic activity in açaí pulp has been found (De Souza et al., 2012). Some hypolipidaemic effects and an improvement in the serum lipid profile have also been described for noni (Lin et al., 2012). Consequently, it has been demonstrated that maqui, açaí and noni fruits are potent inhibitors of pancreatic lipase *in vitro*, so they may be developed – individually or in synergistic formulations – as natural alternatives for obesity treatment through dietary intervention, even though further *in vivo* research is needed.

3.4. *Antioxidant capacity*

With respect to the DPPH· assays, maqui fruits showed the highest activity against this radical, maqui^{CHI1} and maqui^{CHI2} being the most reactive, followed by açaí samples, due to their high anthocyanin content (Céspedes et al., 2008; Del Pozo-Insfran, Brenes, & Talcott, 2004), as demonstrated by the positive and direct correlation between DPPH· and TAC ($R^2 = 0.816^{**}$, $P < 0.001$). To a lesser degree, Cape gooseberry calyx^{COL}, papaya^{ECU} and noni^{ECU}, exhibited DPPH· scavenging activity. It is important to emphasize that the Cape gooseberry calyx^{COL} was more active than the berry, with regard to this activity (Table 4).

The values obtained for the fruits in the ORAC-Fl assay ranged from to 30.36 3.29 mM Trolox/100 mg dw: açaí^{COL}, maqui^{CHI1} and açaí^{BRZ1} – in decreasing order – showed the highest activities (Table 4). All fruits exhibited great scavenging activity except Cape gooseberry fruit^{COL}, which again showed much lower activity than the calyx. The açaí, maqui, noni and papaya fruits had higher ORAC values than over 100 different kinds of foods, including fruits, vegetables, nuts, dried fruits, spices and cereals from the United States (Wu et al., 2004). Hence, they represent a promising source of antioxidant compounds.

Regarding the ABTS·$^+$ assay, the maqui and açaí berries were expected to be the most-active fruits according to the results of DPPH·, but açaí^{BRZ2} gave a low value, while maqui, with the highest TAC, were the most active. Indeed, the TAC and ABTS·$^+$ values were highly correlated ($R^2 = 0.781^{***}$, $P < 0.001$). Noni^{ECU} and Cape gooseberry calyx^{COL} exhibited good antioxidant capacities, probably owing to their contents of flavonol (kaempferol) derivatives (Table 2).

Fruits rich in anthocyanins were very active in FRAP assay, where maqui^{CHI1} had the highest value, followed by maqui^{CHI2}, maqui^{CHI3}, açaí^{BRZ1} and açaí^{COL} (Table 4), also supported by a strong correlation between the FRAP and TAC values ($R^2 = 0.840^{***}$, $P < 0.001$). Moreover, the FRAP assay values were strongly correlated with DPPH· ($R^2 = 0.928^{***}$, $P < 0.001$), ORAC ($R^2 = 0.811^{***}$, $P < 0.001$) and ABTS·$^+$ ($R^2 = 0.917^{***}$, $P < 0.001$). As in the rest of the antioxidant methods, Cape gooseberry calyx^{COL} showed better activity than the fruit.

The superoxide radical anion plays an important role in the formation of other ROS, such as hydrogen peroxide, hydroxyl radical and singlet oxygen, which induce oxidative damage in lipids, proteins and DNA (Gülçin, 2006). Low IC_{50} values were obtained for the Latin-American fruits (Table 4), suggesting high activities against this ROS, except for Cape gooseberry fruit^{COL} and papaya^{ECU}. The values ranged between 0.67 and 36.43 mg/mL dw, for maqui^{CHI1} and papaya^{ECU}, respectively (Table 4).

As seen from these results, the Latin-American fruits tested can effectively scavenge different types of ROS or free radicals under *in vitro* conditions. The broad range of activities of the fruits indicates that multiple mechanisms may be responsible for the antioxidant activity, linked to their characteristic phenolic compounds (Gironés-Vilaplana, Valentão, et al., 2012). In fact, the fruits with high quantities of anthocyanins (açaí and maqui) exhibited higher activities in all the assays, suggesting that flavonoid glycosides, namely anthocyanins (Del Pozo-Insfran et al., 2004), kaempferol derivatives (Han et al., 2004) and flavonols (Cos et al., 1998; Pulido, Bravo, & Saura-Calixto, 2000), in the natural complex matrix of the fruits may be involved in these actions. These high antioxidant effects of maqui and açaí have also previously been reported (Araya, Clavijo, & Herrera, 2006; Céspedes et al., 2008; Gironés-Vilaplana, Mena, et al., 2012; Gironés-Vilaplana, Valentão, et al., 2012; Schauss et al., 2006). Noni fruit also displayed good activity against the superoxide radical, as previously reported (Calzuola, Luigi Gianfranceschi, & Marsili, 2006), although it was below that of maqui and açaí. Moreover, the bioactivity of these fruits was influenced by extraction procedure, concentration, variety and part of the fruit, origin, genotype, ripening and industrial processing (Speisky, López-Alarcón, Gómez, Fuentes, & Sandoval-Acuña, 2012). The results of certain antioxidant assays and the anti-diabesity effects were somewhat poorly correlated (for example, $R^2 = -0.665$, $P < 0.001$ between lipase and DPPH·, and $R^2 = -0.683$, $P < 0.001$ and $R^2 = -0.640$, $P < 0.001$ between α-glucosidase and ORAC and ABTS·$^+$, respectively). This suggests that some flavonoids can react distinctly against oxidative radicals with respect to their action at the active site of α-glucosidase and pancreatic lipase, but others, such as anthocyanins, may display simultaneous antioxidant and anti-diabesity effects.

4. Conclusions

The phytochemical profiling of fruits presented in this study revealed a diverse range of bioactive phenolics and biological activities. Regarding their potential biological activity, maqui was the best-performing fruit in terms of α-glucosidase and lipase inhibition. Papaya showed high α-glucosidase inhibition and noni fruits also exhibited significant lipase inhibition. The maqui and açaí berries were the most-interesting fruits in terms of antioxidant capacity, due to their high anthocyanin (cyanidin and delphinidin derivatives) contents. Noni fruits and Cape gooseberry calyx also exhibited good antioxidant capacities. The value of these Latin-American fruits as valuable sources of phytochemicals for food product development is clear, regarding nutrition and new dietary options for the treatment of diseases such as obesity and diabetes. Further, *in vivo* research will be conducted, to allow scientifically-backed statements and recommendations for dietary intake to be made.

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Phytochemistry and biological activity of Spanish *Citrus* fruits

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The evaluation of the potential inhibitory activity on α-glucosidase and pancreatic lipase by *Citrus* spp. fruits of Spanish origin (lemon, orange, grapefruit, lime, and mandarin) together with the evaluation of their phytochemical content and antioxidant capacity (DPPH•, ORACFL, $ABTS^{+}$, FRAP and $O_2^{\bullet-}$) aiming for new applications of the fruits in nutrition and health was carried out. As far as we are aware, the presence of 3-*O*-caffeoylferuoylquinic acid and two hydrated feruloylquinic acids in orange and the presence of 3,5-diferuoylquinic acid in grapefruit have been reported for the first time. Although grapefruit showed higher contents of phytochemicals such as flavanones and vitamin C, lemon and lime showed higher potential for inhibitory effects on lipase, and lime also showed the best results for *in vitro* α-glucosidase inhibition. On the other hand, higher antioxidant capacity was reported for grapefruit, lemon and lime, which correlated well with their phytochemical composition. Based on the results, it could be concluded that *Citrus* fruits are of great value for nutrition and treatment of diet-related diseases such as obesity and diabetes, and consequently, a new field of interest in the food industry regarding new bioactive ingredients would be considered.

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1. Introduction

It has been strongly demonstrated that the increasing trend in obesity is accompanied by a growing incidence of diabetes. The inhibition of pancreatic lipase in the case of obesity, and of α-glucosidase in the case of diabetes, is the current therapeutic approach for the treatment of both diseases, since these enzymes play an essential role in lipid and glucose metabolisms.[1,2] In this sense, some *Citrus* fruits represent a good source of bioactive compounds with certain antidiabetic and lipolytic effects,[3,4] which are being studied nowadays with increasing interest.

Citrus fruits are among the most important horticultural crops, and are consumed mostly as fresh products or juices because of their nutritional value and special flavour. The total *Citrus* production in Spain was 5 773 619 tonnes in 2011,[5] making it the sixth largest producer in the world after Brazil, China, the U.S., Mexico, and India. Oranges (*Citrus sinensis* L.) are widely produced in Spain and in most parts of the world, but significant quantities of lemons (*Citrus limon* Burm. f), grapefruits (*Citrus paradisi*, Macfad), mandarins (*Citrus reticulata* Blanco), and limes (*Citrus aurantifolia* Christm.) are also grown. It has been strongly demonstrated that these *Citrus* species are thought to possess beneficial effects on health due to their phytochemical composition, mainly flavonoids and vitamin C, having promising prospects in prevention of diseases such as obesity, diabetes, cardiovascular diseases, neurodegenerative disorders and certain types of cancer,[3,6–10] and in the lowering of blood lipid levels.

Some of these *Citrus* fruits and juices have also been used as functional foods and drinks with potential application in the treatment of diet-related diseases in people with different health conditions.[11–13] However, as far as we know, there are no reports in the literature regarding a comprehensive comparison of the enzymatic effects and antioxidant capacity of different *Citrus* fruits rich in polyphenols in the same study. Hence, the aim of this work is to evaluate the antidiabetic and antilipolytic effects (α-glucosidase and lipase inhibitory effects) of 5 *Citrus* whole fruits (lemon, orange, grapefruit, lime, and mandarin) of Spanish origin providing a thorough description on the polyphenolic composition (flavones, flavanones, hydroxycinnamic acids and vitamin C), and correlate it with their antioxidant capacity (DPPH•, $ORAC_{FL}$, $ABTS^{+}$, FRAP and $O_2^{\bullet-}$).

2. Results and discussion

2.1. Phenolic compounds

The HPLC-DAD-ESI/MSn analysis of the hydromethanolic extract of *Citrus* fruits revealed a wide range of different phytochemicals with flavones and flavanones being the major compounds (Table 1). Hydroxycinnamic acids were also present (Table 1).[14] According to molecular masses, fragmentation patterns, characteristic spectra, and bibliographical sources,[15–17] the following

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Table 1 Bioactive composition (flavones, flavanones and hydroxycinnamic acid derivatives) identified and quantified in *Citrus* fruits (mg per 100 g dry weight)

Compound	Rt	$[M-H]^-$	MSn	Lemon	Orange	Lime	Mandarin	Grapefruit
Flavanones (280 nm)								
FV1 *O*-tyglycosil-naringenin	22.3	741	579, 279	—	28.57 ± 0.81	—	78.41 ± 1.53	33.37 ± 4.79
FV2 eriodictyol 7-*O*-rutinoside	30.9	595	577, 287	938.30 ± 16.45	68.05 ± 2.68	257.34 ± 0.47	—	—
FV3 naringenin 7-*O*-rutinoside	37.7	579	271	—	139.90 ± 0.13	48.80 ± 0.20	488.81 ± 14.08	309.26 ± 4.63
FV4 naringenin 7-*O*-neohesperidoside	42.2	579	271	—	—	—	—	2530.65 ± 26.77
FV5 hesperitin 7-*O*-rutinoside	45.8	609	301	372.89 ± 4.03	335.55 ± 5.80	188.54 ± 1.24	123.59 ± 2.38	—
FV6 isosakuranetin-7-*O*-rutinoside	61.4	593	285	—	—	—	319.92 ± 15.39	226.84 ± 0.06
Total				1311.19 ± 20.48	572.07 ± 3.80	494.67 ± 1.91	1010.73 ± 0.46	3100.11 ± 26.86
Flavones (360 nm)								
FL1 apigenin-6,8-di-*C*-glucoside	22.8	593	503, 473	45.99 ± 0.19	19.60 ± 0.74	65.38 ± 0.93	50.13 ± 0.39	10.96 ± 0.93
FL2 luteolin-7-neohesperidoside 4-*D*-glucoside	26.3	756	623, 594, 286	22.32 ± 1.56	8.70 ± 2.17	12.05 ± 0.65	—	—
FL2 diosmetin-6,8-di-*C*-glucoside	28.1	623	503, 413, 383	63.58 ± 0.21	6.27 ± 0.29	44.88 ± 1.34	5.61 ± 0.51	—
FL4 diosmetin-7-*O*-rutinoside	44.8	607	299	21.56 ± 0.21	3.39 ± 0.43	13.35 ± 0.46	—	—
FL5 limocitrin-3-rutinoside	47.7	653	345	21.32 ± 0.11	—	11.34 ± 2.85	—	16.22 ± 0.34
Total				174.76 ± 2.07	37.96 ± 0.72	147.00 ± 3.43	55.74 ± 0.11	27.17 ± 0.33
Hydroxycinnamic acid derivatives (320 nm)								
HA1 4-*O*-coumaroylquinic acid	5.7	337	173	—	21.41 ± 0.01	—	—	—
HA2 dicaffeoylquinic acid (1)	6.1	515	353	—	21.40 ± 0.36	24.49 ± 2.15	23.64 ± 0.66	12.63 ± 0.71
HA3 dicaffeoylquinic acid (2)	7.0	515	353	—	—	31.36 ± 0.72	32.92 ± 1.24	18.62 ± 2.26
HA4 3-*O*-caffeoyl-4-*O*-feruoylquinic acid	8.2	530	513	—	26.40 ± 0.06	—	—	—
HA5 3-*O*-feruoylquinic acid hydrated	9.6	385	367, 173	—	21.01 ± 0.03	—	—	—
HA6 3-*O*-caffeoylquinic acid	12.3	353	191, 179	62.65 ± 0.32	14.60 ± 0.39	44.90 ± 0.32	69.01 ± 0.35	41.65 ± 0.70
HA7 5-*O*-caffeoylquinic acid	16.1	353	191	18.52 ± 0.73	55.09 ± 0.17	6.44 ± 0.06	57.10 ± 4.50	87.66 ± 1.95
HA8 5-*O*-feruoylquinic acid hydrated	18.9	385	367, 173	—	—	—	—	29.09 ± 0.04
HA9 ferulic acid	19.2	175	169	25.78 ± 0.02	—	—	—	—
HA10 sinapic acid	21.3	447	285	25.03 ± 0.83	—	—	—	—
HA11 3,5-*O*-diferuoylquinic acid	32.0	561	367, 173	—	—	—	58.81 ± 1.10	—
Total				131.98 ± 1.26	188.99 ± 0.65	106.98 ± 2.61	241.48 ± 5.64	160.56 ± 1

flavanones were identified: *O*-tryglycosil-naringenin, eriodictyol-7-*O*-rutinoside (eriocitrin), naringenin-7-*O*-rutinoside (narirutin), naringenin-7-*O*-neohesperidoside (naringin), hesperitin-7-*O*-rutinoside (hesperidin), and isosakuranetin-7-*O*-rutinoside (didymin). Grapefruit has a higher quantity of total flavanones, mainly represented by exceptional amounts of naringin, previously reported in the fruit.[18] Higher flavanone amounts were also reported for lemon and mandarin fruits, with eriocitrin being the major flavanone in lemon, and narirutin being the major flavanone in mandarin. It is important to emphasize the role of these bioactive compounds in maintaining health, since they are directly related to anti-inflammatory activities, anticancer effects, and prevention of atherosclerosis, among others.[19]

With respect to flavones, apigenin-6,8-di-*C*-glucoside, luteolin-7-neohesperidoside 4-D-glucoside, diosmetin-6,8-di-*C*-glucoside, diosmetin-7-rutinoside and limocitrin-3-rutinoside were identified, in concordance with previous reports.[16] Lemon and lime fruits have all the flavones identified, and higher amounts of total and individual flavones (Table 1). Apigenin-6,8-di-*C*-glucoside and diosmetin-6,8-di-*C*-glucoside were the major flavones found in lemon and lime fruits, while apigenin-6,8-di-*C*-glucoside is the major flavone in orange and also in mandarin. Grapefruit, which has a lesser quantity of flavones compared to that of flavanones, has been reported to contain only the flavones apigenin-6,8-di-*C*-glucoside and limocitrin-3-rutinoside. These flavones have been previously described to play an important role in the prevention of cancer and cardiovascular diseases.[20]

Several hydroxycinnamic acid derivatives were also detected in *Citrus* species, some of them for the first time, according to MS data and fragmentation patterns: 4-*O*-coumaroylquinic acid, two compounds tentatively identified as isomers of dicaffeoylquinic acid (with MS^- 515, and MS_2 353), 3-*O*-caffeoylferuoylquinic acid, 3-*O*-feruoylquinic acid hydrated (with MS^- 367 + 18 = 385), 3-*O*-caffeoylquinic acid, 5-*O*-caffeoylquinic acid, 5-*O*-feruoylquinic acid hydrated, ferulic acid, sinapic acid, and 3,5-*O*-diferuoylquinic acid. It is important to emphasize that, to date, this is the first available report on the presence of 3-*O*-caffeoylferuoylquinic acid and both hydrated feruoylquinic acids in orange, and of 3,5-diferuoylquinic acid in grapefruit. As far as we are aware, hydrated forms of feruoylquinic acid do not exist in nature, so probably these compounds were hydrated during the extraction procedure, by a hydromethanolic extract. The orange fruit has the largest amount of hydroxycinnamic acids, while mandarin is richest in total derivatives (Table 1). These hydroxycinnamic acids and their derivatives have been demonstrated to possess *in vitro* and *in vivo* antioxidant activities.[21]

2.2. Total Phenolic Compounds (TPC) by the Folin–Ciocalteu reagent

TPC results are expressed as mg per 100 mL of gallic acid equivalents (GAE). *Citrus* fruits have been reported to contain large quantities of TPC in the decreasing order as follows: grapefruit (202.36 ± 1.32), lemon (180.73 ± 5.93), orange (104.05 ± 9.31), mandarin (100.57 ± 1.87), and lime (94.78 ± 1.86). However, TPC values must be interpreted with caution since the Folin–Ciocalteu reagent can react not only with phenolic compounds, but also with a variety of non-phenolic reducing compounds including tertiary aliphatic amines, amino acids (tryptophan), hydroxylamine, hydrazine, certain purines, and other organic and inorganic reducing agents leading to an overestimation of the phenolic contents. Furthermore, different phenolic compounds may have different responses to the Folin–Ciocalteu reagent, presenting lower absorption, which results in the underestimation of compounds,[22] so these results should be evaluated together with those obtained by the analysis of phenolic compounds by HPLC-DAD-ESI/MSn (Table 1).

2.3. Vitamin C

It has been widely demonstrated that *Citrus* fruits possess significant amounts of vitamin C, as the sum of ascorbic acid (AA) and dehydroascorbic acid (DHAA).[23] AA, DHAA and the total vitamin C content (AA + DHAA) of *Citrus* fruits are expressed in mg per 100 g (Table 2). Grapefruit with the highest amounts of AA, DHAA and total vitamin C was separated from the rest of the *Citrus* fruits with significantly lower quantities of AA, DHAA and total vitamin C. These differences between *Citrus* fruits according to the variety can also be induced by all the factors that affect the vitamin C and AA content including cultural practice, maturity, climate, fresh fruit handling, processing factors, blanching, packaging, and storage conditions.[23] These results of ascorbic and dehydroascorbic acid contents were noticeably higher than those previously reported for other fruits or vegetables, like Sweet Pepper (*Capsicum annuum* L.).[24] Furthermore, *Citrus* fruits with higher TPC content are also reported to have more vitamin C ($R^2 = 0.782$**, $P < 0.01$). Ascorbic acid is known for a number of vital biological activities including synthesis of collagen, neurotransmitters, steroid hormones, and carnitine, and is responsible for the conversion of cholesterol to bile acid.[25] Apart from this, other major clinical investigations were conducted to understand the benefits of ascorbic acid in prevention of the common cold, iron absorption, ulcers, colorectal carcinoma, hypertension, prevention of atherosclerosis, and advanced malignancy.[26] Therefore, *Citrus* fruits represent good sources of vitamin C, associated with beneficial effects on health.

Table 2 Ascorbic acid (AA), dehydroascorbic acid (DHAA) and total vitamin C (AA + DHAA) content of *Citrus* fruits (mg per 100 g dried product)[a]

Citrus fruit	AA	DHAA	Vitamin C
Lemon	57.66 ± 5.12[a]	97.60 ± 9.20[a]	155.26 ± 14.32[a]
Orange	84.82 ± 0.86[b]	36.24 ± 0.96[a]	121.06 ± 0.50[a]
Lime	81.57 ± 3.82[ab]	46.70 ± 0.52[a]	128.27 ± 3.30[a]
Mandarin	64.03 ± 5.54[ab]	67.26 ± 8.79[a]	131.29 ± 14.33[a]
Grapefruit	114.52 ± 10.81[c]	283.19 ± 34.87[b]	397.71 ± 42.51[b]
LSD $P < 0.05$	6.162	22.791	28.411

[a] Means ($n = 3$) in the same columns followed by different letters are significantly different at $P < 0.05$ according to Tukey's test.

Table 3 Antioxidant capacity, α-glucosidase inhibition, and lipase activity of *Citrus* fruits[a]

Fruit	DPPH•	ABTS+	FRAP	ORAC	$O_2^{\bullet-}$	α-Glucosidase	Lipase
	mmol Trolox per 100 g d.w.	mmol Trolox per 100 g d.w.	mmol Trolox per 100g d.w.	mmol Trolox per 100 g d.w.	IC_{50} (mg mL^{-1})	IC_{50} (mg mL^{-1})	U L^{-1}
Lemon	3.92 ± 0.11^{c}	9.00 ± 0.58^{c}	7.53 ± 1.02^{a}	31.26 ± 3.42^{b}	1.33 ± 0.20^{a}	36.59 ± 1.60^{b}	93.74 ± 7.39^{a}
Orange	1.54 ± 0.20^{a}	4.83 ± 0.17^{a}	5.95 ± 1.11^{a}	19.44 ± 0.30^{a}	1.79 ± 0.27^{a}	—	186.89 ± 6.75^{b}
Lime	2.53 ± 0.15^{b}	6.14 ± 0.59^{ab}	7.35 ± 1.37^{a}	45.12 ± 3.49^{c}	1.54 ± 0.12^{a}	10.96 ± 0.31^{a}	111.37 ± 4.17^{a}
Mandarin	2.50 ± 0.17^{b}	6.47 ± 0.30^{b}	5.13 ± 0.33^{a}	31.70 ± 2.50^{b}	2.96 ± 0.03^{b}	—	182.20 ± 8.62^{b}
Grapefruit	4.22 ± 0.19^{c}	8.69 ± 0.42^{c}	7.07 ± 0.20^{a}	46.33 ± 1.77^{c}	2.54 ± 0.25^{b}	62.10 ± 2.32^{c}	179.10 ± 13.14^{b}
LSD $P < 0.05$	0.182	0.424	0.756	2.109	0.160	1.035	6.973

[a] Means ($n = 3$) in the same columns followed by different letters are significantly different at $P < 0.05$ according to Tukey's test. * Samples without data did not inhibit 50% of the enzyme.

2.4. Antioxidant capacity

The antioxidant capacity of *Citrus* fruits was tested against different reactive species: DPPH•, $ORAC_{FL}$, $ABTS^{+}$, FRAP and $O_2^{\bullet-}$. DPPH• and $ABTS^{+}$ are non-biological radicals extensively used to test the antioxidant capacity of plant samples. Other widespread methods for the evaluation of the antioxidant capacity of vegetal samples are FRAP, based on the reduction of Fe; and ORAC, based on the peroxyl radical scavenging ability. Free radicals, like $O_2^{\bullet-}$, are produced in the body as a result of aerobic metabolism, playing an important role in the formation of other reactive species that result in a wide array of biological damage in living cells.[27] So the use of various methods can provide a more complete evaluation of the antioxidant capacity of the *Citrus* fruits.

2.4.1. DPPH•. DPPH• is one of the few stable and commercially available organic nitrogen radicals and has an UV-vis absorption maximum at 515 nm. Upon reduction, the color of the solution fades; and the reaction progress is conveniently monitored using a spectrophotometer.[28] According to DPPH• results, it was found that lemon and grapefruit have the highest activity against this radical, followed by lime and mandarin ($P < 0.05$, Table 3). Lemon and grapefruit also displayed higher amounts of flavanones and vitamin C, showing a positive and direct correlation between DPPH• and these phytochemical contents ($R^2 = 0.793$***, $P < 0.001$ for flavanones, and $R^2 = 0.726$*, $P < 0.01$ for vitamin C). Moreover DPPH• was strongly correlated with TPC ($R^2 = 0.900$***, $P < 0.001$). All *Citrus* fruits presented significantly lower activities when compared to other antioxidant assays performed as the values in the DPPH• results were lower than in the $ABTS^{+}$ and FRAP assays. Previously, the best DPPH• scavenger among the four *Citrus* fruits (lemon, orange, lime and grapefruit) was lime,[29] in disagreement with these results, showing that the other plant extracts exhibit strong antiradical activity too, with an IC_{50} between 0.36 and 0.99 μg extract per mL.[30]

2.4.2. $ABTS^{+}$. The free radical scavenging ability of plant samples is also studied using a moderately stable nitrogen-centred radical species: the $ABTS^{+}$ radical.[31] All the tested *Citrus* fruits exhibited a significant activity, showing similar results to those obtained by the DPPH• method. The details regarding the scavenging assays are as follows: lemon and grapefruit exhibited the highest scavenging activity, and orange the lowest (Table 3). For this reason, we found a strong and direct correlation between the results of these two antioxidant methods ($R^2 = 0.956$***, $P < 0.001$). Moreover, the total flavanone content played a significant role in antiradical activity ($R^2 = 0.698$**, $P < 0.01$), with the *Citrus* fruits having a higher quantity of flavanones being the most reactive. $ABTS^{+}$ was also correlated with TPC ($R^2 = 0.874$***, $P < 0.001$). Previous studies showed high antioxidant properties of *Citrus* peel phenolic extracts, like grapefruits, against $ABTS^{+}$, which might be useful in the formulation of nutraceuticals and food preservatives.[31]

2.4.3. FRAP (ferric reducing antioxidant power). The FRAP method is used to measure the total reducing capability of antioxidants based on their potential to react with the ferric tripyridyltriazine (Fe^{3+}–TPTZ) complex and produce a blue colour due

to the ferrous form, which can be detected with the absorbance peak at 593 nm.[32] The tested *Citrus* fruits displayed a high and very similar antioxidant capacity ($P < 0.05$) in the FRAP assay (Table 3), as expected;[33,34] but lemon and grapefruit, as in DPPH$^{\cdot}$ and ABTS^{+} tests, and lime exhibited higher activity than orange and mandarin. These FRAP results were higher than those previously reported for an Italian saffron (*Crocus sativus* L.).[35] Other juices from *Citrus* varieties cultivated in China also reported to have high activity.[36] No significant correlation between FRAP and any other test was found, which suggested the different mode of action in this method based on iron reduction, with the previous radical scavenging assays employed.

2.4.4. $ORAC_{FL}$. The $ORAC_{FL}$ assay provides a direct measure of hydrophilic chain-breaking antioxidant capacity against the peroxyl radical.[37] The values obtained for the fruits in the $ORAC_{FL}$ assay varied distinctly among the samples (ranged between 19.44 and 46.33 mM Trolox/100 mg dw (Table 3)), with lime and grapefruit being the most reactive samples in this case, followed by lemon and mandarin, and orange being reported with the lowest value again ($P < 0.05$). It is interesting to know that *Citrus* fruits showed higher ORAC values than over 100 different kinds of foods, including fruits, vegetables, nuts, dried fruits, spices, and cereals from the United States.[38]

2.4.5. Superoxide radical ($O_2^{\cdot -}$). The superoxide anion ($O_2^{\cdot -}$) plays an important role in the formation of other ROS such as hydrogen peroxide (H_2O_2), singlet oxygen (O_2), and hydroxyl radicals (OH^-), which induce oxidative damage in lipids, proteins, and DNA. These species are produced by a number of enzyme systems in autooxidation reactions and by nonenzymatic electron transfer that univalently reduce molecular oxygen.[39] Concerning the $O_2^{\cdot -}$ scavenging results, low IC_{50} values were obtained (Table 3), suggesting a high activity of the *Citrus* fruits against this reactive oxygen species, among which lime, lemon, and orange were the most active. In fact, total flavones were correlated with $O_2^{\cdot -}$ scavenging activity ($R^2 = -0.720$**, $P < 0.01$). Flavones and flavanones of *Citrus* flavonoids have been described as good superoxide scavengers,[40] supporting this strong effect. A point worth mentioning is that although all *Citrus* fruits were very active against the $O_2^{\cdot -}$ radical, some differences between this method and the rest were found, probably due to the differences in the mode of action of this biological method compared to the rest of the chemical radicals.

Citrus fruits can effectively scavenge different types of reactive oxygen species or free radicals under *in vitro* conditions (Table 3). The broad range of results indicates that multiple mechanisms may be responsible for their antioxidant capacity, related to their phenolic composition, mainly flavones and flavanones, and their vitamin C content. Although all the antioxidant methods have a different nature and origin between them, *Citrus* fruits followed a similar trend in all the methods in general, suggesting that grapefruit, lemon and lime are the most antioxidant rich fruits among all the samples used in this study, and orange and mandarin exhibited lower antioxidant activity. In summary, the combination of phytochemicals and synergistic mechanisms in the fruit matrix is highly responsible for the potent antioxidant activities of fruits.

2.5. α-Glucosidase inhibition

α-Glucosidase is a key enzyme that catalyses carbohydrates in the final step of the digestive process. Therefore the inhibition of this enzyme could delay the digestion of oligosaccharides and disaccharides to monosaccharides, which in turn reduces glucose absorption and consequently reduces postprandial hyperglycemia.[2] The IC_{50} values were calculated in order to compare the different *Citrus* fruits, as shown in Table 3. Different effects were observed as follows: orange and mandarin did not cause the 50% inhibition of the enzyme, while lemon and grapefruit caused slight inhibition, with lime being more effective (Fig. 2). Flavones and flavanones were reported to be potent α-glucosidase inhibitors.[41] Moreover, some *Citrus* flavonoids, like hesperidin, naringin and polymethoxylated flavones, have demonstrated potential benefits in the management of diabetes in some animal models by different biochemical mechanisms.[4] The IC_{50} values and total vitamin C (AA + DHAA) were strongly correlated ($R^2 = 0.879$***, $P < 0.001$), but no Pearson correlations between any flavonoid groups and anti-α-glucosidase activity were found in our results. The α-glucosidase inhibitory activities were consistent with the statement that different phytochemical profiles and the interactions between compounds in the fruit matrix can also be involved in various activities displayed by them. Lime and lemon fruits are active against α-glucosidase, with lime having the highest inhibitory effect among all the *Citrus* fruits analyzed. Thus, lime fruit may offer dietary coadjuvants to control hyperglycemia in diabetic patients, however further research in the evaluation of their *in vivo* antidiabetic activity is needed to verify this beneficial effect.

2.6. Pancreatic lipase inhibition

The inhibition of pancreatic lipase, which splits triglycerides into absorbable glycerol and fatty acids, is the main prescribed treatment for obesity in developed countries.[1,42] In order to find alternative sources for obesity prevention and treatment, we searched for the inhibitory action of the Spanish *Citrus* fruits on lipase activity. The results are shown in Table 3 and Fig. 1 as U L^{-1} and % of inhibition of the lipase enzyme, respectively;

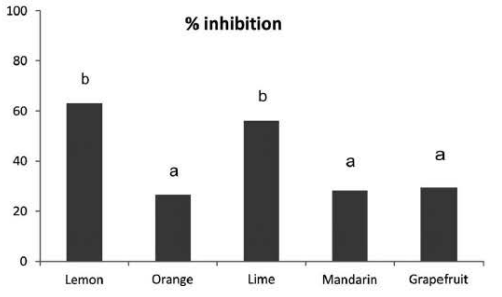

Fig. 1 Lipase inhibition (%) of Spanish *Citrus* fruits (100 mg dried fruit per 1 mL of methanol–water 70 : 30 v/v).

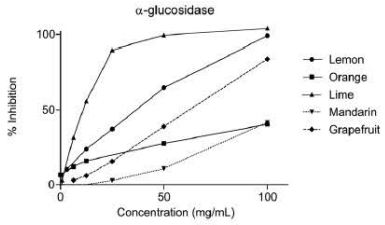

Fig. 2 α-Glucosidase inhibition of Spanish *Citrus* fruits.

taking into consideration that the activity of the lipase standard was 260 U L^{-1}. Lemon and lime fruits displayed the highest inhibitory effect on pancreatic lipase (93.74 and 111.37 U L^{-1}, respectively), being also richer in flavones as seen above, and finding a strong correlation between % of lipase inhibition and the total flavone content ($R^2 = 0.969$***, $P < 0.001$). This potent inhibitory activity of flavones on the lipase enzyme has been previously reported.[43] Moreover, citric acid has also been described as a driver of thermogenesis, reducing obesity risk.[44] The other citrus fruits namely orange, mandarin and grapefruit that were previously demonstrated to improve the lipid metabolism with some of their phytochemicals, such as eriocitrin[8] or hesperitin,[45] also displayed certain inhibitory effects. Consequently, *Citrus* fruits of Spanish origin, especially lemon and lime, have demonstrated *in vitro* inhibition of pancreatic lipase. Taking into account that lime and lemon were also the best performing fruits in terms of α-glucosidase inhibition, they may be developed individually or in synergistic formulations as natural alternatives for the treatment of obesity and diabetes through dietary intervention, even though further *in vivo* research is needed.

3. Experimental

3.1. Chemicals

2,2-Diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$), 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt (ABTS^{+}), 2,4,6-tripyridyl-*s*-triazine (TPTZ), ferric chloride hexahydrate, fluorescein (free acid), 2,2′-azobis(2-methyl-propionamidine) dihydrochloride (APPH), sodium phosphate monobasic, sodium phosphate dibasic, Folin–Ciocalteu reagent, β-nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), nitrotetrazolium blue chloride (NBT), trizina hydrochloride, 4-nitrophenyl α-D-glucopyranoside, α-glucosidase from *Saccharomyces cerevisiae*, and potassium phosphate were obtained from Sigma-Aldrich (Steinheim, Germany). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and magnesium chloride hexahydrate were purchased from Fluka Chemika (Neu-Ulm, Switzerland); anhydrous sodium carbonate, sodium benzoate and potassium sorbate were bought from Panreac Química S.A. (Barcelona, Spain). LIPASE-PS™ (Kit) was obtained from Trinity Biotech (Jamestown, NY, USA). Ultrapure water was produced using a Millipore water purification system.

3.2. Fruits

Citrus fruits were purchased at 'Carrefour Planet' Store (Centros Comerciales Carrefour S.A., Murcia), gathered from different producers:

- Lemon: *C. limon* (Burm. f), lemon cv. 'verna' (caliber 3/4-58/72 mm; Cat. 1.; Los Ramos, Murcia)
- Orange: *C. sinensis* (L.) (Cat. 1.; Los Ramos, Murcia)
- Lime: *C. aurantifolia* (Christm.) Swingle, lime. (CIF B-29607751, Málaga)
- Mandarin: *C. reticulata* (Blanco), Honey tangerine cv. 'Murcott' (caliber 54/64 mm, Piles, Valencia)
- Grapefruit: *C. paradisi* (Macfad), 'Star Ruby' red grapefruit (caliber 84/97 mm; Cat. 1, Castellón, Valencia)

3.3. Extraction

All whole fruits were cut into 3 cm portions, frozen with liquid N_2 and freeze dried. An amount of 100 mg of sample was weighed and added to 1 mL of methanol–water (70 : 30% v/v). Then, the samples were vortexed and sonicated in an ultrasonic bath for 60 min. Samples were kept at 4 °C overnight, and sonicated again for 60 min. A centrifugation (model EBA 21, Hettich Zentrifugen) step (10 000 rpm, 5 min) was used to separate the supernatant from the solid residue. This supernatant was filtered through a 0.45 μm PVDF filter (Millex HV13, Millipore, Bedford, Mass., USA) and stored at 4 °C before performing all analytical methods. Three different extractions were made for each method.

3.4. Identification of phenolic compounds by HPLC-DAD-ESI/MSn and quantification by RP-HPLC-DAD

Chromatographic analyses for the identification were carried out on a Luna C18 column (250 × 4.6 mm, 5 mm particle size; Phenomenex, Macclesfield, UK) in an Agilent HPLC 1100 series equipped with a photodiode array detector and a mass detector in series (Agilent Technologies, Waldbronn, Germany) with the same conditions used previously by Gironés-Vilaplana *et al.*[46] The equipment consisted of a binary pump (model G1312A), an autosampler (model G1313A), a degasser (model G1322A) and a photodiode array detector (model G1315B). The HPLC system was controlled by ChemStation software (Agilent, version 08.03).

For the quantification, a HPLC-DAD system was used, as described by Gironés-Vilaplana *et al.*[47] Different phenolic compounds were characterised by chromatographic comparison with analytical standards as well as quantified by the absorbance of their corresponding peaks. Flavonols and flavones were quantified as quercetin-3-*O*-glucoside at 360 nm, cinnamic acids as 5-*O*-caffeoylquinic acid at 320 nm, and flavanones as hesperidin at 280 nm.

3.5. Total Phenolic Compounds (TPC) by the Folin–Ciocalteu reagent

The Folin–Ciocalteu reagent method was adapted to a microscale assay according to ref. 48. The results were expressed as mg per 100 mL of gallic acid equivalents (GAE).

3.6. Extraction and analysis of vitamin C

The vitamin C content was determined by HPLC as described by González-Molina *et al.*[49] AA (ascorbic acid) and DHAA (dehydroascorbic acid) were identified and quantified by comparison with pattern areas from AA and DHAA. The vitamin C content was calculated by adding AA and DHAA contents, and the results were expressed as mg per 100 g dry weight.

3.7. Antioxidant capacity

The free radical scavenging activities were determined using the DPPH$^{\bullet}$, ABTS^{+} and FRAP (ferric reducing antioxidant power) methods adapted to a microscale according to Mena *et al.*[50] The antioxidant activity was evaluated by measuring the variation in absorbance at 515 nm after 50 min (DPPH$^{\bullet}$), at 414 nm after 50 min (ABTS^{+}) of reaction with the radical, and finally at 593 nm after 40 min for the FRAP assay. Assays were performed by using 96-well microplates (Nunc, Roskilde, Denmark) and an Infinite® M200 microplate reader (Tecan, Grödig, Austria). All reactions were started by adding 2 μL of the corresponding diluted sample to the well containing the stock solution (250 μL). The final volume of the assay was 252 μL. The antioxidant activity was also determined using the ORAC-FL assay, according to Ou *et al.*[37] Results were expressed as mM Trolox per 100 mg dry weight.

The superoxide radical ($O_2^{\bullet -}$) scavenging activity was also determined spectrophotometrically using a 96-well plate reader by monitoring the effect of controls and blends on the $O_2^{\bullet -}$ induced reduction of NBT at 560 nm. Superoxide radicals were generated by the NADH/PMS system according to a described procedure.[51] The experiments were performed in triplicate, and the results were expressed in IC_{50} (concentration of the sample to inhibit 50% of the radicals).

3.8. α-Glucosidase inhibitory activity

The α-glucosidase inhibitory activity was assessed by modification of a previously reported procedure.[52] Briefly, each well contained 100 μl of 2 mM 4-nitrophenyl α-D-glucopyranoside in 10 mM potassium phosphate buffer (pH 7.0) and 20 μL of the samples, diluted 1/2 in buffer. The reaction was initiated by the addition of 100 μL of the enzyme solution (56.66 mU mL^{-1}). The plates were incubated at 37 °C for 10 min. The absorbance of 4-nitrophenol released from 4-nitrophenyl α-D-glucopyranoside at 400 nm was measured. The increase in absorbance was compared with that of the control (buffer instead of sample solution) to calculate the inhibitory activity and IC_{50}.

3.9. Lipase inhibitory effect

The lipase inhibitory activity was determined as previously described by Gironés-Vilaplana *et al.*,[46,47] and adapted to a microscale on 96-well microplates (Nunc, Roskilde, Denmark) using an Infinite® M200 microplate reader (Tecan, Grödig, Austria). The recorded rate of increase in absorbance at 550 nm due to the formation of quinone diimine dye was used to determine the pancreatic lipase activity in the samples prepared. The pancreatic lipase activity in fruits was expressed in U L^{-1}.

3.10. Statistical analysis

Data presented are mean values ($n = 3$) ± standard deviation. All data were subjected to analysis of variance (ANOVA) and a Multiple Range Test (Tukey's test), using IBM SPSS statistics 21 software (SPSS Inc., Chicago, IL). Pearson's correlation analysis was performed to corroborate relationships between selected parameters.

4. Conclusions

Nowadays, only a limited number of publications dealing with the bioactive composition of *Citrus* fruits and their potential effects on health are found. The hydromethanolic extracts of *Citrus* fruits revealed a wide and diverse range of phytochemicals, mainly flavones, flavanones, and vitamin C (AA + DHAA), and significant antioxidant capacity and biological activity. Grapefruit displayed the highest phytochemical contents in terms of flavanones and vitamin C. To the best of our knowledge, this is the first available report of 3-*O*-caffeoylferuoylquinic acid and both hydrated feruoylquinic acids in orange, and of 3,5-diferuoylquinic acid in grapefruit. Although grapefruit, lemon and lime performed better in terms of antioxidant capacity, which correlated well with flavanones and vitamin C, lemon and lime are the best candidates for antidiabetic and antilipolytic purposes (α-glucosidase and lipase inhibition), which also correlated with vitamin C and flavone contents, respectively. Therefore multiple biological activities indicate the potential of lemon and lime *Citrus* to be sources of bioactive compounds for new product developments (*i.e.* combinations of fruits to enrich new foods or beverages), with potential applications in diet-related diseases such as obesity and diabetes. However, more *in vivo* research and safety evaluations should be performed to allow scientifically backed statements and recommendations for dietary intake.

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2. PUBLICATIONS 3, AND 4: DESIGN OF NEW BEVERAGES MADE OF LEMON JUICE AND DIFFERENT FRUITS POWDERS AND CONCENTRATES. CHARACTERIZATION OF THEIR PHYTOCHEMICAL CONTENT, ANTIOXIDANT CAPACITY AND ANTICHOLINERGIC EFFECTS

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Phytochemical profile of a blend of black chokeberry and lemon juice with cholinesterase inhibitory effect and antioxidant potential

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ABSTRACT

In this study, black chokeberry concentrate was added (5% w/v) to lemon juice, since previous reports suggested potential health benefits of this blend. The phytochemical composition, antioxidant capacity (scavenging of DPPH, superoxide and hydroxyl radicals, and hypochlorous acid), and inhibitory activity against cholinesterase of the new blend were determined and compared with those of lemon juice and chokeberry in citric acid (5%). The chokeberry concentrate, rich in cyanidin-glycosides, quercetin derivatives, and 3-*O*-caffeoylquinic acid, and lemon juice, possessing flavones, flavanones, quercetin derivates, and hydroxycinnamic acids, were characterised. The new drink showed a higher antioxidant effect than the chokeberry or lemon controls for all the tested methods, except for hypochlorous acid, in which lemon juice displayed higher activity. Both the lemon juice and chokeberry controls inhibited acetylcholinesterase and butyrylcholinesterase, and this effect was increased in the new mixtures. The results of the different radical scavenging assays indicate that the lemon–black chokeberry (5% w/v) mixture was more antioxidative than the respective controls separately. Moreover, their inhibition of cholinesterase is of interest regarding neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, or senile dementia.

1. Introduction

There is increasingly strong scientific evidence that a diet rich in fruits and vegetables may reduce the risk of suffering different chronic diseases (Borges, Mullen, & Crozier, 2010). The results obtained with the techniques employed in the past were insufficient to support this relationship, so it was necessary to examine more thoroughly the characteristics of the food in order to identify possible metabolites responsible for these preventive effects. Consequently, the current trend is to study foods in order to demonstrate that this relationship between consumption and prevention is correct. An easy and convenient form of consuming fruits is in juice form, and one focus of current research is the design of fruit-based beverages rich in phytochemicals and the evaluation of their bioactivity.

Citrus fruits are among the most important horticultural crops, lemon (*Citrus limon* (L.) Burm. f.) being the third most important *Citrus* crop species (González-Molina, Domínguez-Perles, Moreno, & García-Viguera, 2010). Several studies have pointed out that lemon is a rich source of nutrients and phytochemicals, including flavonoids, citric acid, vitamin C, and minerals (González-Molina, Moreno, & García-Viguera, 2008a), which have numerous health promoting properties (González-Molina et al., 2010; Mulero et al., 2012). For this reason, lemon juice is an interesting food matrix for designing new beverages, as well as being a suitable source of value-added products since overproduction and non-marketable fruits lead to a serious environmental problem of unused agrowaste on a yearly basis. In this regard, lemon juice represents an alternative for the conversion of a bioburden into a food product.

Black chokeberry (*Aronia melanocarpa* (Michx.) Elliott) is a natural, rich source of phenolic antioxidants, such as cyanidin 3-*O*-glycoside anthocyanins (cyanidin 3-*O*-galactoside, cyanidin 3-*O*-glucoside, cyanidin 3-*O*-arabinoside, and cyanidin 3-*O*-xyloside) (González-Molina, Moreno, & García-Viguera, 2008b), quercetin derivatives (Bermúdez-Soto & Tomás-Barberán, 2004), hydroxycinnamic acids (Zheng & Wang, 2003), and, in smaller amounts, vitamin C (Benvenuti, Pellati, Melegari, & Bertelli, 2004; Skupien & Ozmianski, 2007). Many reports have suggested its anti-proliferative effects against cancer cells (Lala et al., 2006), as well as antimutagenic (Gasiorowski, Szyba, Brokos, Kolaczynska, Jankowiak-Wlodarczyk & Oszmianski, 1997), hepatoprotective (Kowalczyk et al., 2004), cardioprotective (Hellström et al., 2010),

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and antidiabetic activities (Valcheva-Kuzmanova, Kuzmanov, Tancheva, & Belcheva, 2007). Moreover, a series of papers reported the antioxidant properties of black chokeberry extracts or their phenolic constituents, using various, well established *in vitro* and *in vivo* models for direct antioxidant capacity (Bermúdez-Soto & Tomás-Barberán, 2004; Rop et al., 2010; Zheng & Wang, 2003), as well as their protective effects against oxidative stress (Kedzierska, Olas, Wachowicz, Stochmal, Oleszek & Erler, 2011). Recently, the neuroprotective effects of cyanidin-3-*O*-glycosides, commonly present in black chokeberry, have been tested in mice (Min et al., 2011).

In the regulation of cognitive functions, the central cholinergic system is considered to be the most important neurotransmitter involved (Mukherjee, Kumar, Mal, & Houghton, 2007). In addition, cholinesterases, such as acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE), are key enzymes that play important roles in cholinergic transmission, by hydrolysing the neurotransmitter acetylcholine (Vinholes et al., 2011). Cholinergic neuronal loss in the hippocampal area is the major feature of Alzheimer's disease (AD), senile dementia, ataxia, myasthenia gravis, and Parkinson's disease, and inhibitors of AChE and BuChE are the pharmacological treatment used for these diseases (Mukherjee et al., 2007). These drugs have adverse side-effects, like gastrointestinal disturbances, nausea, vomiting, and diarrhoea, as well as problems of bioavailability. Because of this, researchers are seeking natural AChE and BuChE inhibitors with a better safety profile. A wide range of plant compounds with this inhibitory activity have been found, mainly alkaloids (Mukherjee et al., 2007), xanthones (Brühlmann, Marston, Hostettmann, Carrupt, & Testa, 2004), and flavonols, such as quercetin (Khan et al., 2009), among others. To the best of our knowledge, no previous work has been published on lemon or black chokeberry compounds as inhibitors of cholinesterases.

Following previous research on lemon juice enriched with black chokeberry concentrate (González-Molina et al., 2008b), the aims of this work were to perform a deeper phytochemical characterisation of this new blend, to extend our knowledge of its antioxidant capacity, and to evaluate its potential with respect to inhibition of cholinesterases, for future nutrition and health uses.

2. Material and methods

2.1. Chemicals

The reagents used were commercially available: 2,2-diphenyl-1-picrylhidracyl radical (DPPH·), β-nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), nitrotetrazolium blue chloride (NBT), trizina hydrochloride, bovine albumin, sodium chloride, acetylcholinesterase from electric eel, butyrylcholinesterase from equine serum, acetylthiocholine iodide, S-butyrylthiocholine chloride, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), sodium borohydride, sodium hypochlorite solution, ferrium chloride (45% solution), 2-deoxy-D-ribose, and 2-thiobarbituric acid were obtained from Sigma–Aldrich (Steinheim, Germany); potassium dihydrogen phosphate, ethylenediaminetetraacetic acid (EDTA), hydrogen peroxide (30%), and trichloroacetic acid were purchased from Merck (Darmstadt, Germany); magnesium chloride hexahydrate and ascorbic acid were bought from Fluka Chemika (Neu-Ulm, Switzerland). Ultrapure water was produced using a Millipore water purification system.

2.2. Samples

Black chokeberry concentrate (62.3 °Brix; pH 3.58) was provided by "Juver Alimentación S.A." (Churra, Murcia, Spain). Lemon juice was obtained, from 'Fino' lemons freshly collected from the CEBAS-CSIC experimental farm ('La Matanza', Santomera, Murcia, SE Spain; 38°6′14″ N, 1°1′59″ W), using a domestic squeezer ('Citromatic', Braun Española S.A., Barcelona, Spain). The juice was stored frozen (−20 °C) until use.

2.3. Experimental design

Black chokeberry concentrate was added to a volume of lemon juice to obtain a final concentration of 5% w/v (g/ml) in the beverage. In addition, a control solution using the same proportion in 0.18 M citric acid buffer (pH 2.46) was prepared, to study the activities of the concentrate without lemon. Lemon juice alone was also assayed (pH 2.14).

The homogenised mixtures and control solutions were centrifuged (7 min at 4000 rpm). The juices were stored frozen (−20 °C) until use.

The samples were labelled as follows: L (lemon juice control), LA (lemon juice plus 5% black chokeberry concentrate), A (5% black chokeberry concentrate in citric acid buffer).

2.4. HPLC-DAD-ESI/MSn

The chromatographic analyses were carried out on a Luna C18 column (250 × 4.6 mm, 5 mm particle size; Phenomenex, Macclesfield, UK). Water:formic acid (99:1, v/v) and acetonitrile were used as the mobile phases A and B, respectively, with a flow rate of 1 ml/min. The linear gradient started with 8% of solvent B, reaching 15% at 25 min, 22% at 55 min, and 40% at 60 min, which was maintained up to 70 min. The injection volume was 30 µl. Chromatograms were recorded at 280, 320, 360, and 520 nm for hydroxycinnamic acids, non-coloured flavonoids, and anthocyanins. The HPLC-DAD-ESI/MS*n* analyses were carried out in an Agilent HPLC 1100 series machine equipped with a photodiode array detector and a mass detector in series (Agilent Technologies, Waldbronn, Germany). The equipment consisted of a binary pump (model G1312A), an autosampler (model G1313A), a degasser (model G1322A), and a photodiode array detector (model G1315B). The HPLC system was controlled by ChemStation software (Agilent, version 08.03). The mass detector was an ion trap spectrometer (model G2445A), equipped with an electrospray ionisation interface and controlled by LCMSD software (Agilent, version 4.1). The ionisation conditions were 350 °C and 4 kV, for capillary temperature and voltage, respectively. The nebulizer pressure and nitrogen flow rate were 65.0 psi and 11 L/min, respectively. The full-scan mass covered the range from 100 to *m*/*z* 1200. Collision induced fragmentation experiments were performed in the ion trap using helium as the collision gas, with voltage ramping cycles from 0.3 up to 2 V. The mass spectrometry data were acquired in the positive ionisation mode for anthocyanins and in the negative ionisation mode for other flavonoids. The MS*n* was carried out in the automatic mode on the more abundant fragment ion in MS($n-1$).

Prior to injection, the samples were centrifuged (12000 rpm, 5 min) and filtered through a PVDF Filter (0.22 µm). Anthocyanins were quantified as cyanidin 3-*O*-glucoside at 520 nm, flavonols as quercetin 3-*O*-rutinoside (rutin) at 360 nm, hydroxycinnamic acids as 5-*O*-caffeoylquinic acid at 320 nm, flavanones as hesperidin at 280 nm, and flavones as diosmin at 360 nm.

2.5. DPPH radical scavenging activity

The antiradical capacity was estimated spectrophotometrically in a Multiskan Ascent plate reader (Thermo Electron Corporation), by monitoring the disappearance of DPPH· at 515 nm, according to Oliveira et al. (2010). Three experiments were performed in triplicate.

2.6. Superoxide radical ($O_2^{\cdot-}$) scavenging activity

This antiradical activity was determined spectrophotometrically, in a 96-well plate reader, by monitoring the effect of the controls and the new drink on the $O_2^{\cdot-}$ induced reduction of NBT at 560 nm. Superoxide radicals were generated by the NADH/PMS system according to a described procedure (Ferreres, Fernandes, Oliveira, Valentão, Pereira & Andrade, 2009). The experiments were performed in triplicate.

2.7. Hypochlorous acid scavenging activity

The inhibition of the hypochlorous acid-induced oxidation of 5-thio-2-nitrobenzoic acid (TNB) to 5,5′-dithiobis(2-nitrobenzoic acid) was studied according to a described procedure (Valentão, Fernandes, Carvalho, Andrade, Seabra & Bastos, 2002), in a double beam spectrophotometer (Heλios α, Unicam, Leeds, UK) at 412 nm. Three experiments were performed in triplicate.

2.8. Hydroxyl radical assay

The deoxyribose method for determining the scavenging effect of samples on hydroxyl radicals was performed as described before (Valentão et al., 2002), in a double beam spectrophotometer (Heλios α, Unicam, Leeds, UK) programmed in photometric function, with the wavelength fixed at 532 nm. Three experiments were performed in triplicate.

2.9. AChE and BChE inhibitory activity

The inhibition of AChE activity was determined based on Ellman's method, as reported previously (Ferreres, Taveira, Pereira, Valentão, & Andrade, 2010). The absorbance was measured at 405 nm and the rates of reaction were calculated using Ascent Software version 2.6 (Thermo Labsystems Oy). The BuChE inhibition assay was performed in a similar way, using 25 μl of substrate (15 mM butyrylthiocholine) and 25 μl of enzyme (0.1 U/ml). Three experiments were performed in triplicate.

2.10. Statistical analyses

The data shown are mean values ($n = 3$). All data were subjected to analysis of variance (ANOVA) and a Multiple Range Test (Tukey's test), using PASW Statistics 18 software (Somers, New York, USA).

3. Results and discussion

3.1. Phenolic compounds

The HPLC-DAD study of the lemon juice (L) and black chokeberry (A) controls revealed the presence of a wide range of polyphenols. Some of them were recently identified by our group (González-Molina et al., 2008b; Mellisho et al., 2011). However, additional phenolic compounds have been identified and quantified in black chokeberry, including cyanidin-glycosides (A1–A4) (Table 1), quercetin derivatives (F1–F6) (Table 1), and one hydroxycinnamic acid, which, by UV spectra and *m/z*, corresponded to 3-*O*-caffeoylquinic acid (C1) (Table 1). Lemon juice flavones, flavanones, quercetin derivatives, and hydroxycinnamic acids were also characterised (Table 1).

In lemon juice control, flavones and flavanones were the major flavonoids, followed by hydroxycinnamic acids and quercetin derivatives (Table 2). Flavones were represented mainly by diosmetin 6,8-di-*C*-glucoside (L2) (55.9%), followed by diosmetin 7-*O*-rutinoside (L4) (36.9%) and apigenin 6,8-di-*C*-glucoside (**L1**) (7.1%) (Table 2). With respect to flavanones, eriodictyol 7-*O*-rutinoside (**L10**) and hesperetin 7-*O*-rutinoside (**L11**) were present at very similar levels, each compound representing almost one-half of the total flavanones (48.8 and 51.2%, respectively) (Table 2). Ferulic acid was the predominant hydroxycinnamic acid (**L8**) (50.7%), followed by sinapic acid (**L9**) (33.5%) and 5-*O*-caffeoylquinic acid (**L7**) (15.8%) (Table 2). These compounds have been characterised previously in lemon juice (Mellisho et al., 2011). However, lower concentrations of flavanones and higher amounts of flavones were found when compared to previous results for "Fino" lemon juice (González-Molina et al., 2008a). It is important to emphasise that the concentration of flavonoids in lemons is variable according to the cultivar, maturity stage, or growth conditions (González-Molina et al., 2010).

The black chokeberry control had considerable amounts of total anthocyanins, quercetin derivatives, and 3-*O*-caffeoylquinic acid (Table 2). Cyanidin 3-*O*-galactoside (**A1**) was the most abundant anthocyanin (66.7% of the total), followed by cyanidin 3-*O*-arabinoside (**A3**) (26.9%) (Table 2). Cyanidin 3-*O*-glucoside (**A2**) and cyanidin 3-*O*-xyloside (**A4**) were present in significantly lower amounts (3.1% and 3.3%, respectively) (Table 2). Concerning quercetin derivatives, all the compounds identified displayed similar concentrations. Quercetin 3-*O*-galactoside (F5) was the most abundant (31%), followed by the pair quercetin 3-*O*-rhamnosyl-galactoside plus quercetin 3-*O*-rhamnosyl-glucoside (26.1%) (F3 + F4) (Table 2).

In relation to the blend (LA), the anthocyanins concentration provided by the black chokeberry concentrate was similar to that of the controls (Table 2). The total hydroxycinnamic acids concentration was higher than in the L and A controls (Table 2). Nonetheless, the flavonols provided by both lemon and chokeberry, and the flavones and flavanones provided by lemon, were lower (Table 2). It should be noted that in the new blend (LA), the lemon phytochemicals were slightly diluted with respect to the lemon control, due to the addition (5%) of chokeberry concentrate. Although some phytochemicals were less abundant in the new beverage, a positive effect was observed regarding biological activities, probably because the matrix was richer in bioactive compounds (anthocyanins, flavonols, hydroxycinnamic acids, flavones, and flavanones). In previous research on lemon juice enriched with black chokeberry concentrate (González-Molina et al., 2008b), the levels of bioactive compounds in the controls and the blends were about one-third of those reported here, as before the addition of the black chokeberry concentrate, it was reconstituted to obtain the °Brix of a commercial juice; so, the concentration was 1/3 (w/v) (González-Molina et al., 2008b). Therefore, there is potential for the use of berry concentrates in the food industry to develop new beverages with increased concentrations of phytochemicals, in addition to the advantages of using less volume of fresh fruit or fruit products.

3.2. Antioxidant activity

The antioxidant activity of all the samples was measured as the scavenging of different radicals: DPPH$^{\cdot}$, $O_2^{\cdot-}$, hypochlorous acid (HOCl), and hydroxyl ($^{\cdot}$OH). All of these are considered valid assays for the evaluation of the antioxidant capacity of plants and foods. The IC_{50} values were calculated, in order to compare the different samples and methods (Table 3).

Concerning DPPH$^{\cdot}$, the lemon juice control showed the lowest activity. The black chokeberry concentrate had a lower IC_{50}; hence, its addition to lemon juice resulted in a high scavenging activity of the blend (Table 3). In previous work (González-Molina et al., 2008b), the addition of 5% black chokeberry concentrate to lemon juice did not increase the antioxidant activity with respect to the control, in contrast to our study (Table 3). Another, commercial black chokeberry juice concentrate had higher activity (60 mg

Table 1
Identification of different bioactive compounds present in chokeberry control and lemon juice.

Compounds	Rt	[M−H]*	MSn	(Lambda) (max)
Black chokeberry				
Anthocyanins (520 nm)				
A1 Cyanidin 3-*O*-galactoside	17.1	449	287	520
A2 Cyanidin 3-*O*-glucoside	19.3	449	287	520
A3 Cyanidin 3-*O*-arabinoside	21.4	419	287	520
A4 Cyanidin 3-*O*-xyloside	26.7	419	287	520
Flavonols (360nm)				
F1 Quercetin diglucoside	29.2	625	463, 301	260, 360
F2 Quercetin pentosilhexoside	34.5	595	433, 301	260, 360
F3 Quercetin 3-*O*-rhamnosyl-galactoside	38.6	609	301	260, 360
F4 Quercetin 3-*O*-rutinoside	39.1	609	301	260, 360
F5 Quercetin 3-*O*-galactoside	40.4	463	301	260, 360
F6 Quercetin 3-*O*-glucoside	41.5	463	301	260, 360
Chlorogenic acid derivatives (320 nm)				
C1 3- caffeoylquinic acid	8.9	353	191, 179	330
LEMON				
Flavones (360 nm)				
L1 Apigenin 6,8-di-*C*-glucoside	24.1	593	503, 473	270, 345
L2 Diosmetin 6,8-di-*C*-glucoside	28.0	623	503, 413, 383	270, 340
L3 8-*C*-glucosilchrysoeriol	42.1	461	300	280, 350
L4 Diosmetin 7-*O*-rutinoside	52.5	607	299	280, 345
Flavonols (360nm)				
L5 Quercetin 3-*O*-rutinoside-7-*O*-glucoside	19.7	771	609, 301	265, 365
L6 Quercetin 3-*O*-rutinoside	38.4	609	301	265, 365
Hydroxycinnamic acid derivatives (320 nm)				
L7 5-caffeoylquinic acid	16.5	353	191	330
L8 Ferulic acid	19.1	175	169	330
L9 Sinapic acid	20.6	205	189	330
Flavanones (280 nm)				
L10 Eriodictyol-7-*O*-rutinoside	34.3	595	287	280
L11 Hesperetin-7-*O*-rutinoside	50.1	609	301	280

Rt: Retention time in minutes.
[M−H]*: Ionisation of masses: Positive for anthocyanins, negative for non-coloured flavonoids.

Table 2
Quantification (mg/100 ml) of different phenolic compounds of the lemon juice control, chokeberry control, and new blend.

Compounds	L	A	LA
Anthocyanins (520 nm)			
A1 Cyanidin 3-*O*-galactoside	–	40.84 ± 0.04	39.97 ± 2.02
A2 Cyanidin 3-*O*-glucoside	–	1.87 ± 0.00	1.90 ± 0.02
A3 Cyanidin 3-*O*-arabinoside	–	16.51 ± 0.01	16.33 ± 0.32
A4 Cyanidin 3-*O*-xyloside	–	2.03 ± 0.01	2.16 ± 0.15
Total anthocyanins	–	61.25 ± 0.06	60.36 ± 2.21
Flavonols (360nm)			
L5 Quercetin 3-*O*-rutinoside-7-*O*-glucoside	nq	–	nq
F1 Quercetin diglucoside	–	2.31 ± 0.06	1.61 ± 0.01
F2 Quercetin pentosilhexoside	–	3.34 ± 0.20	3.22 ± 0.12
F3 Quercetin 3-*O*-rhamnosyl-galactoside	–	5.69 ± 0.19*	5.02 ± 0.11*
F4 Quercetin 3-*O*-rutinoside	0.97 ± 0.04		
F5 Quercetin 3-*O*-galactoside	–	6.76 ± 0.69	5.14 ± 0.15
F6 Quercetin 3-*O*-glucoside	–	3.73 ± 0.29	2.83 ± 0.08
TOTAL FLAVONOLS	0.97 ± 0.04	21.82 ± 1.64	18.57 ± 0.46
Flavones			
L1 Apigenin 6,8-di-*C*-glucoside	0.90 ± 0.10	–	1.10 ± 0.01
L2 Diosmetin 6,8-di-*C*-glucoside	7.02 ± 0.11	–	6.24 ± 0.05
L3 8-*C*-glucosilchrysoeriol	nq	–	nq
L4 Diosmetin 7-*O*-rutinoside	4.64 ± 1.64	–	3.38 ± 0.08
Total flavones	12.55 ± 1.85	–	10.72 ± 0.13
Hydroxycinnamic acid derivatives			
C1 3-caffeoylquinic acid	–	12.47 ± 0.24	18.02 ± 0.07
L7 5-caffeoylquinic acid	0.28 ± 0.00	–	0.41 ± 0.02
L8 Ferulic acid	0.91 ± 0.00	–	1.25 ± 0.02
L9 Sinapic acid	0.60 ± 0.01	–	0.67 ± 0.03
Total hydroxicinnamic acids	1.79 ± 0.01	12.47 ± 0.24	20.34 ± 0.12
Flavanones			
L10 Eriodictyol-7-*O*-rutinoside	3.72 ± 0.09	–	3.67 ± 0.03
L11 Hesperetin-7-*O*-rutinoside	3.90 ± 0.38	–	3.48 ± 0.08
Total flavanones	7.63 ± 0.47	–	7.16 ± 0.11

Trolox/ml) (Bermúdez-Soto & Tomás-Barberán, 2004), as did dried fruits, pomace, or other juices (279.38, 301.84, and 127.45 μmoles Trolox/100 g dry weight, respectively) (Oszmiański & Wojdylo, 2005). The DPPH scavenging effect has been reported also for fruit

Table 3
Antioxidant and anticholinesterase (AChE and BuChE) activities of lemon juice, black chokeberry, and lemon plus black chokeberry*.

	DPPH·	$O_2^{\cdot-}$	HOCl	·OH	AChE	BuChE
L	13.27^c	5.16^b	25.71^a	6.93^c	13.18^a	12.82^b
A	5.27^b	6.42^c	59.48^c	4.90^b	12.97^a	18.98^c
LA	4.23^a	3.24^a	28.95^b	3.74^a	10.57^a	10.89^a
LSD, $p < 0.05$	0.24	0.23	0.27	0.17	1.12	0.44

* IC_{50} values are expressed in mg/ml. L: Lemon juice, A: 5% black chokeberry concentrate in citric acid control, LA: 5% black chokeberry concentrate in lemon juice. Means ($n = 3$) in the same columns followed by different letters are significantly different at $P < 0.05$ according to Tukey's test.

extracts (IC_{50} = 1.8 mg/ml, and 181.07 μmol TE/g) (Benvenuti et al., 2004; Jakobek, Šeruga, Novak, & Medvidović-Kosanović, 2007) and for black chokeberry snacks (IC_{50} = 10.04 mg/ml) (Gramza-Michałowska & Człapka-Matyasik, 2011), the latter being less effective than the samples tested herein.

Previous reports described a direct correlation between the DPPH• scavenging activity and the total anthocyanins content of anthocyanin-rich fruit extracts (Espín, Soler-Rivas, Wichers, & García-Viguera, 2000) and selected or red fruits (Dragović-Uzelac, Levaj, Bursać, Pedisić, Radojčić & Biško, 2007; Jakobek et al., 2007). In five black chokeberry cultivars, a direct correlation between DPPH• scavenging and cyanidin 3-*O*-arabinoside and cyanidin 3-*O*-galactoside, also identified in our study, was found (Rop et al., 2010). Nonetheless, no significant correlation existed when the total flavone glycosides of citrus varieties were compared with the DPPH· scavenging capacity (Xu, Liu, Chen, Ye, Ma & Shi, 2008). In accordance with these results, the higher activity of samples containing black chokeberry concentrate (A, LA) can be related to their levels of anthocyanins.

The controls (L and A) showed low values of IC_{50} for superoxide ($O_2^{\cdot-}$) scavenging assay, indicative of a high activity against this radical. Moreover, the LA blend was more effective than the controls (Table 3). The polyphenols present in both lemon juice and black chokeberry are potent $O_2^{\cdot-}$ scavengers (Yu, Wang, Walzem, Miller, Pike & Patil, 2005), including rutin, apigenin, and quercetin (Masuoka, Matsuda, & Kubo, 2012). It is reasonable to suppose that these flavonoid glycosides with more than one en-diol group, like quercetin or apigenin, chemically reduced this radical and decreased the superoxide generation (Masuoka et al., 2012). On the other hand, anthocyanin extracts from rambutan (*Nephelium lappaceum* L.) or litchi (*Litchi chinenesis* Sonn.) showed excellent superoxide anion scavenging activity (IC_{50} = 415.8 μg/ml and a scavenging activity of 91.4% in a 50 μg/ml sample, respectively) (Duan, Jiang, Su, Zhang, & Shi, 2007; Sun, Peng, Su, Yao, Long & Wang, 2011). Scavenging activity of black chokeberry fruit against the superoxide anion has been reported previously (Rop et al., 2010). In summary, the anti-radical effect of the samples against $O_2^{\cdot-}$ may be due to their contents of non-coloured flavonoids, such as rutin, quercetin, or apigenin, and anthocyanins, since this effect was more pronounced in the blend, which was richer in these compounds.

The activity against HOCl was lower. The IC_{50} values varied significantly, between 25.6 and 59.5 mg/ml (Table 3). The black chokeberry control was far less active than lemon juice; hence, the addition of black chokeberry concentrate to lemon juice did not improve its activity (Table 3). Other work has demonstrated that citrus pulp might be an effective HOCl scavenger (Ramful, Tarnus, Aruoma, Bourdon, & Bahorun, 2011) and showed a significant correlation between the total phenolic content of citrus extracts and their scavenging of HOCl. This is consistent with the slightly greater effect of lemon juice in comparison with the blends (Table 3). A weak protective effect against HOCl was described also for cardoon (*Cyanara cardunculus* L.) infusion (Valentão et al., 2002) and beefsteak fungus (IC_{15} = 1.46 mg/ml) (*Fistulina hepatica*) extract (Ribeiro, Valentão, Baptista, Seabra, & Andrade, 2007). No effect was found with *Linaria vulgaris* (Vrchovská, Spilková, Valentão, Sousa, Andrade & Seabra, 2008). However, other plant species showed activity against the oxidative species; for example, *Centaurium erythraea* (IC_{25} = 0.93 mg/ml) (Valentão, Fernandes, Carvalho, Andrade, Seabra & Bastos, 2003).

Regarding the hydroxyl radical (·OH), the low IC_{50} values indicate good antioxidant capacity (Table 3). The black chokeberry control displayed better activity than the lemon juice control, as reported also for methanolic extracts of black chokeberry (Rop et al., 2010). As was found for DPPH and $O_2^{\bullet-}$, the LA blend was the most potent sample. Anthocyanin extracts from pomegranate (*Punica granatum*) (Noda, Kaneyuki, Mori, & Packer, 2002) and litchi (Duan et al., 2007) showed ·OH scavenging activity, mainly due to chelation by metal ions rather than by direct scavenging of the radical. However, ascorbic acid from lemon juice may act as a pro-oxidant because of its ability to affect the redox cycling of the metal ion required for the hydroxyl generation, thus increasing the radical production (Valentão et al., 2002). The maximum vitamin C content of black chokeberry was reported to be 30 mg/100 g fw (Benvenuti et al., 2004; Skupien & Ozmianski, 2007), but no vitamin C was found in our chokeberry samples, presumably owing to the thermal treatment used for the industrial concentration. Nevertheless, no pro-oxidant effect was observed in the lemon juice, probably because the antioxidant potential of the lemon flavonoids offsets this effect.

So, the addition of black chokeberry concentrate to lemon juice enhanced its antioxidant activity against the tested reactive species, except HOCl. The results suggest that the polyphenol content is not the only reason for the antioxidant capacity; also, the quality and the interactions between matter (Gramza-Michałowska & Człapka-Matyasik, 2011).

3.3. Inhibition of AChE and BuChE

In recent years, the search for inhibitors of cholinesterases has grown in interest, since these enzymes are associated with Alzheimer's disease, senile dementia, ataxia, myasthenia gravis, and Parkinson's disease, among others (Mukherjee et al., 2007; Vinholes et al., 2011). *In vitro* enzyme studies are used to screen for potential bioactivity of certain substances against these enzymes, leading to further studies *in vivo*. The IC_{50} values of the controls (L, A) and the blend (LA) are presented in Table 3 and Fig. 1. All the samples displayed AChE and BuChE inhibitory activities, those of the lemon juice and black chokeberry controls being similar for AChE. In the BuChE assay, the lemon juice displayed a higher activity than the concentrate (Table 3 and Fig. 1). The LA blend had the strongest activity against both cholinesterases (Table 3 and Fig. 1). The flavonols of lemon and black chokeberry, and the presence of *C*-glycosyl flavones in lemon, which have been demonstrated to be cholinesterase inhibitors (Khan et al., 2009), may have been responsible for this. This effect was considerably lower than that of the pharmacological drugs used to treat neurological diseases, such as galantamine (IC_{50} for AChE = 0.14 μg/ml) (Wszelaki, Kuciun, & Kiss, 2010) or huperzine A ($IC_{50} = 10^{-4}$ μM) (Mukherjee et al., 2007). However, this new blend represents a natural alternative, which can be taken every day, without side effects. It should be noted also that it is not a purified extract. The IC_{50} values of other natural products and extracts have been reported to range between 29 and 178 μg/ml (Wszelaki et al., 2010), while commercial oils (IC_{50} = 0.015–3.2 mg/ml; Dohi, Terasaki, & Makino, 2009) and tomato seeds (IC_{20} = 2.4 mg/ml; Ferreres et al., 2010) have been tested also. Recently, the inhibition of AChE and BuChE by a hydroxymethanolic extract of *Spergularia rubra* was tested

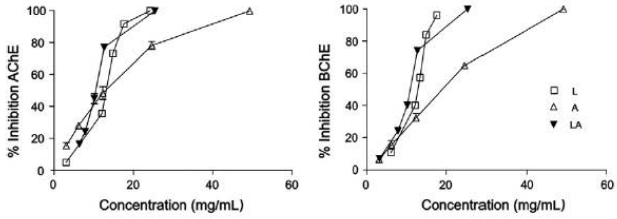

Fig. 1. Acetylcholinesterase and butyrylcholinesterase inhibitory activity of the samples: Lemon juice (L), black chokeberry control (A), and lemon juice plus 5% black chokeberry concentrate (LA). Each point represents the mean of the values obtained from three independent experiments, in each of which triplicate samples were analysed.

(IC_{25} = 3.68 mg/ml and 4.29 mg/ml for AChE and BChE, respectively) (Vinholes et al., 2011), showing activity only slightly greater than our samples.

Our results are very promising, because until now cholinesterase inhibitory activity of lemon juice or black chokeberry has not been reported, as far as we know.

4. Conclusions

A new blend of lemon juice and black chokeberry concentrate was characterised with respect to its functional activity and phytochemical profile. The results of different radical scavenging assays indicate that the lemon–black chokeberry (LA) mixture (5% w/v, black chokeberry concentrate) showed greater antioxidant capacity than the respective controls separately, except for HOCl, against which lemon juice performed better. With respect to cholinesterases, both lemon and black chokeberry displayed inhibitory activity, which increased in the blend. Taking into account the role of cholinesterases and their inhibition in neurodegenerative diseases, these results are of interest regarding natural AChE and BuChE inhibitors within foods. Therefore, this new blend has the potential to be developed into a product with both nutritive and health promoting properties. Further metabolic and biological activity studies are necessary, as well as the search for more sources of bioactive phytochemicals possessing these characteristics.

Acknowledgments

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New Beverages of Lemon Juice Enriched with the Exotic Berries Maqui, Açaí, and Blackthorn: Bioactive Components and in Vitro Biological Properties

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ABSTRACT: Following previous research on lemon juice enriched with berries, the aim of this work was to design new blends based on lemon juice mixed with different edible berries of exotic and national origin: maqui (*Aristotelia chilensis* (Molina) Stuntz), açaí (*Euterpe oleracea* Mart.), and blackthorn (*Prunus spinosa* L.). The phytochemical characterization of controls and blends was performed by HPLC-DAD-ESI/MS^n. Their antioxidant capacity against DPPH, superoxide, and hydroxyl radicals and hypochlorous acid and their potential to inhibit cholinesterases were also assessed. The profiling of the red fruits and lemon revealed a wide range of bioactive phenolics. The novel beverage based on lemon juice and maqui berry (LM) was the most interesting blend in terms of antioxidant capacity. Berry control samples displayed reduced effects on acetylcholinesterase and butyrylcholinesterase, the lemon juice control being always the most active. This activity was also remarkable for lemon−blackthorn (LB) and lemon−açaí (LA) blends, the last being the most effective inhibitor of cholinesterases among all samples. The results suggested that 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.

KEYWORDS: *Aristotelia chilensis, Euterpe oleracea, Prunus spinosa, Citrus limon, antioxidant, cholinesterase inhibition*

INTRODUCTION

In recent years, chronic diseases, including cancer and cardiovascular and neurological disorders, have been taking special relevance in society. For this reason, a continuous flow of information and research results on the positive impact of fruits and vegetables on these diseases has been rising. Previous studies have demonstrated that berries have potential against chronic diseases, and their use in the fresh form or mixed with other juices could act as prevention and improve human health status in these disorders.[1,2]

The loss of basal forebrain cholinergic cells results in an important reduction in acetylcholine, which is believed to play an important role in the cognitive impairment associated with Alzheimer's disease (AD), senile dementia, ataxia, myasthenia gravis, and Parkinson's disease.[3] Taking into account that cholinesterases, such as acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), are the principal enzymes involved in the hydrolysis of acetylcholine, cholinesterase inhibitors are being developed for the treatment of these diseases. In addition, a wide range of plant compounds with cholinesterase inhibitory activity have been found that may be relevant to the treatment of these neurodegenerative disorders.[4] The available studies are focused on alkaloids,[4,5] xanthones,[6] quercetin,[7] and phlorotannins,[8] among others. Rodent models revealed that the polyphenolic compounds found in berries may decrease the risk of developing age-related neurodegenerative diseases,[9] and cyanidin-3-*O*-glucoside, commonly present in berries, displayed neuroprotective effects in mice with focal cerebral ischemia.[10]

Current trends and worldwide developments on new foods and products with functionality aim to demonstrate a significant bioactivity of tropical or exotic berries. In this sense, previous research[1,2] designing beverages by combining lemon juice (*Citrus limon* (L.) Burm. f.) with berries resulted in effectively increased antioxidant properties of the lemon juice, as well as improved organoleptic characteristics of the new beverage and offers new possibilities against health problems of chronic diseases.[11] To the best of our knowledge, no previous work about the potential of *C. limon*, *Aristotelia chilensis* (Molina) Stuntz (maqui), *Euterpe oleracea* Mart. (açaí), and *Prunus spinosa* L. (blackthorn) as AChE and BChE inhibitors has been performed.

Maqui is a common edible berry from central and southern Chile that is a source of natural colorants due to the presence of anthocyanins. This fruit has also been recently reported as one of the healthiest berries, due to its bioactive components.[12,13] Several papers have linked maqui's phenolics with its high antioxidant capacity,[14] in vitro inhibition of adipogenesis and inflammation,[13] protection against oxidative stress,[15] cardioprotection,[12] and in vitro and in vivo antidiabetic effects.[14,16] Açaí is a berry from the palm tree, which is native to the Amazon River area in South America. It is commonly used fresh and in the preparation of beverages and has recently

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become popular as a functional food due to its antioxidant potential and phytochemical composition.[17] Potential benefits have been attributed to açaí fruits, extracts, and juices: antioxidant,[18,19] anti-inflammatory,[19] reduction of selected markers of metabolic disease risk[20] or atherosclerosis,[21] pain reduction and improved mobility,[22] and antiproliferative properties.[23] Blackthorn is a fruit of deciduous shrubs native to Europe, mainly Spain, Portugal, and Turkey.[24] It is commonly used in the preparation of jams or macerated with aniseed liqueur to obtain a digestive alcoholic drink called patxarán. Blackthorn is also cited as an astringent, diuretic, and purgative[24] and has recently been proved to be an antioxidant.[25] However, a characterization of its phenolic composition and potential for health-related benefits remains understudied.

Lemon is usually available as fresh produce and is also widely used in the food industry for the elaboration of juices, lemonades, and other processed products.[26] Nonetheless, large quantities of low-quality and over-ripe fruits not optimal for consumers are wasted as byproduct. In this regard, with the aim of minimizing the impact of this bioburden, new alternatives are needed. Lemon juice is rich in nutrients, including vitamin C, minerals, citric acid, and bioactive flavonoids, which can provide health benefits beyond nutrition on cardiovascular disease, cancer, diabetes, and obesity, among other chronic problems of adulthood.[26]

The aims of this work were to perform a phytochemical characterization of lemon juice, maqui, açaí, and blackthorn berries, to design new blends of lemon juice enriched with the berries (5% w/v), to determine their antioxidant capacity, and to evaluate their potential as cholinesterase inhibitors for future applications in nutrition and health.

MATERIALS AND METHODS

Chemicals. Reagents were commercially available: 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$), β-nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), nitrotetrazolium blue chloride (NBT), trizina hydrochloride, bovine albumin, sodium chloride, acetylcholinesterase from electric eel, butyrylcholinesterase from equine serum, acetylthiocholine iodide, *S*-butyrylthiocholine chloride, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), sodium borohydride, sodium hypochlorite solution, ferrium chloride 45% solution, 2-deoxy-D-ribose, and 2-thiobarbituric acid were obtained from Sigma-Aldrich (Steinheim, Germany); potassium dihydrogen phosphate, ethylenediaminetetraacetic acid (EDTA), hydrogen peroxide 30%, and trichloroacetic acid were purchased from Merck (Darmstadt, Germany); magnesium chloride hexahydrate and ascorbic acid were from Fluka Chemika (Neu-Ulm, Switzerland). Ultrapure water was produced using a Millipore water purification system.

Fruits. Maqui and açaí lyophilized berries were provided by Ecuadorian Rainforest, LLC (USA). Blackthorn fruit was obtained from Importaciones Samanes S.L. and lyophilized and ground after. Lemon juice was obtained from 'Fino' lemons freshly collected from CEBAS-CSIC's experimental farm (La Matanza, Santomera, Murcia, southeastern Spain; 38° 6′ 14″ N, 1° 1′ 59″ W), using a domestic squeezer (Citromatic, Braun Española S.A., Barcelona, Spain). Juice was stored frozen (−20 °C) until used.

Experimental Design. Lyophilized and powdered fruits were added to lemon juice separately to obtain final concentrations of 5% w/v of the fruit in the beverage. In addition, control solutions using 0.18 M citric acid buffer (pH 2.46) were prepared to study the activities of the different fruits without lemon. Lemon juice alone was also assayed (pH 2.15). Homogenized mixtures and control solutions were then centrifuged (7 min at 4000 rpm) and stored frozen (−20 °C) until used.

Samples were labeled as follows: L (lemon juice control), LM 5% (lemon juice plus 5% of maqui berry), M 5% (5% maqui in citric acid buffer), LA 5% (lemon juice plus 5% of açaí berry), A 5% (5% açaí in citric acid buffer), LB 5% (lemon juice plus 5% of blackthorn fruit), B 5% (5% blackthorn in citric acid buffer).

HPLC-DAD-ESI/MS^n. Chromatographic analyses were carried out on a Luna C18 column (250 × 4.6 mm, 5 mm particle size; Phenomenex, Macclesfield, U.K.). Water/formic acid (99:1, v/v) and acetonitrile were used as mobile phases A and B, respectively, with a flow rate of 1 mL/min. The linear gradient started with 8% of solvent B, reaching 15% solvent B at 25 min, 22% at 55, and 40% at 60 min, which was maintained up to 70 min. The injection volume was 30 μL. Chromatograms were recorded at 280, 320, 360, and 520 nm. The HPLC-DAD-ESI/MS^n analyses were carried out in an Agilent HPLC 1100 series equipped with a photodiode array detector and a mass detector in series (Agilent Technologies, Waldbronn, Germany). The equipment consisted of a binary pump (model G1312A), an autosampler (model G1313A), a degasser (model G1322A). and a photodiode array detector (model G1315B). The HPLC system was controlled by ChemStation software (Agilent, version 08.03). The mass detector was an ion trap spectrometer (model G2445A) equipped with an electrospray ionization interface and was controlled by LCMSD software (Agilent, version 4.1). The ionization conditions were adjusted at 350 °C and 4 kV for capillary temperature and voltage, respectively. The nebulizer pressure and flow rate of nitrogen were 65.0 psi and 11 L/min, respectively. The full-scan mass covered the range from m/z 100 to m/z 1200. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas, with voltage ramping cycles from 0.3 to 2 V. Mass spectrometry data were acquired in the positive ionization mode for anthocyanins and in negative ionization mode for other flavonoids. MS^n was carried out in the automatic mode on the more abundant fragment ion in MS$^{(n-1)}$.

Prior to injection, samples were centrifuged (12000 rpm, 5 min) and filtered through a PVDF syringe filter (0.22 μm). Anthocyanins were quantified as cyanidin 3-*O*-glucoside at 520 nm, flavonols as quercetin 3-*O*-rutinoside (rutin) at 360 nm, ellagic acid derivatives as ellagic acid at 360 nm, hydroxycinnamic acids as 5-*O*-caffeoylquinic acid at 320 nm, flavanones as hesperidin at 280 nm, and flavones as diosmin at 360 nm.

DPPH Radical Scavening Activity. The antiradical capacity was estimated spectrophotometrically in a Multiskan Ascent plate reader (Thermo Electron Corp.) by monitoring the disappearance of DPPH$^{\bullet}$ at 515 nm, according to Oliveira et al.[27] The reaction mixtures in the sample wells consisted of 25 μL of samples (five different concentrations to obtain IC_{50}) and 200 μL of DPPH$^{\bullet}$ dissolved in methanol. Three experiments were performed in triplicate.

Superoxide Radical ($O_2^{\bullet-}$) Scavenging Activity. Antiradical activity was determined spectrophotometrically in a 96-well plate reader by monitoring the effect of controls and blends on the $O_2^{\bullet-}$-induced reduction of NBT at 560 nm. Superoxide radicals were generated by the NADH/PMS system according to a described procedure.[28] Samples were tested at five different concentrations to obtain IC_{50}. All components were dissolved in phosphate buffer (19 mM, pH 7.4). Experiments were performed in triplicate, expressing results as inhibition percentage of NBT reduction compared to the control.

Hypochlorous Acid Scavenging Activity. The inhibition of hypochlorous acid-induced 5-thio-2-nitrobenzoic acid (TNB) oxidation to DTNB was performed according to a described procedure,[29] in a double-beam spectrophotometer (Helios α, Unicam, Leeds, U.K.) at 412 nm. Samples are tested at five different concentrations and were diluted in phosphate buffer (50 mM, pH 7.4) to obtain IC_{50}. Three experiments were performed in triplicate.

Hydroxyl Radical Assay. The deoxyribose method for determining the scavenging effect of samples on hydroxyl radicals was performed as described before[29] in a double-beam spectrophotometer (Helios α), programmed in photometric function, with the wavelength fixed at 532 nm. Hydroxyl radical is generated in a Fenton system, in which ascorbic acid accelerates hydroxyl radical formation

by reducing Fe^{3+} ions to Fe^{2+}. Therefore, the radical is detected by its ability to degrade deoxyribose into fragments. The heating of the mixture under acid conditions leads to the formation malonaldehyde. This is detected by its reaction with thiobarbituric acid, with the formation of a pink chromogen: Reaction mixtures contained, in a final volume of 1 mL, 50 μM ascorbic acid, 40 μM $FeCl_3$, 2 mM EDTA, 2.8 mM H_2O_2, 2.8 mM deoxyribose, sample, and 10 mM KH_2PO_4–KOH buffer (pH 7.4) as solvent. Samples were tested at five different concentrations to obtain IC_{50}. Three experiments were performed in triplicate.

AChE and BChE Inhibitory Activity. The inhibition of AChE activity was determined on the basis of Ellman's method, as previously reported.[30] The absorbance was measured at 405 nm, and the rates of reactions were calculated by Ascent Software version 2.6 (Thermo Labsystems Oy). The BChE inhibition assay was performed in a similar way, using 25 μL of substrate (15 mM butyrylthiocholine) and 25 μL of enzyme (0.1 U/mL). Three experiments were performed in triplicate. Each sample was evaluated at five different concentrations to obtain IC_{50}, and three experiments were performed in triplicate.

Statistical Analysis. Data shown are mean values (n = 3). All data were subjected to analyses of variance (ANOVA) and a multiple-range test (Tukey's test), using PASW Statistics 18 software (Somers, NY, USA). Pearson correlation analysis was performed to corroborate relationships between selected parameters.

RESULTS AND DISCUSSION

Phenolic Compounds. The analysis of the berry fruits revealed the presence of a wide range of polyphenols. Concerning maqui, different glycosides and diglycosides of delphinidin (**A1**, **A2**, **A5**, **A6**) and cyanidin (**A3**, **A4**) were found (Figure 1; Table 1), in accordance with previous papers.[2,31] Flavonols (quercetin and myricetin derivatives, **F1**–**F5**, **F7**–**F10**), ellagic acid derivatives (**E2** and **E3**, with maximum absorption at 360 nm), 5-*O*-caffeoylquinic acid (**C6**), and one ellagitannin (granatin B, **E1**, identified and quantified at 320 nm) were also identified (Figure 2; Table 1). Only the maqui sample presented ellagic acid derivatives. With respect to açaí, three derivatives of cyanidin (**A7**, **A8**, **A10**) and one of malvidin (**A14**) were identified (Figure 1; Table 1). Likewise, quercetin (**F5**, **F7**, **F9**, and **F10**) and hydroxycinnamic acid derivatives (**C3**, **C6**, **C8**) were found, too (Figure 2; Table 1), these compounds being also previously reported in açaí.[32,33] Blackthorn presented four anthocyanins (two cyanidin glycosides (**A8**, **A10**) and two peonidin glycosides (**A12**, **A13**)) (Figure 1; Table 1), quercetin derivatives (**F4**, **F6**, **F8**), and hydroxycinnamic acid derivatives (**C1**–**C5**, **C7**) (Figure 2; Table 1), in accordance with previous research.[25] Lemon juice contained flavones, flavanones, flavonols, and hydroxycinnamic acids (Figure 3; Table 2), as described before.[34]

Maqui control (M) and blend (LM) showed considerably high amounts of anthocyanins (Table 3). Delphinidin 3-*O*-sambubioside-5-*O*-glucoside (**A1**), delphinidin 3,5-*O*-diglucoside (**A2**), cyanidin 3,5-*O*-diglucoside (**A3**), and cyanidin 3-*O*-sambubioside-5-*O*-glucoside (**A4**) contributed 49.8% in M and 48.9% in LM of the total anthocyanins amount (Table 3). Delphinidin 3-*O*-glucoside (**A6**) was the second major anthocyanin in maqui samples (22.1 and 21.8%, respectively). This high content in anthocyanins in maqui has been also previously reported.[2,13,31] Blackthorn (B) and açaí (A) controls and blends (LB and LA, respectively) displayed lower quantities of anthocyanins. With respect to açaí, cyanidin-3-*O*-rutinoside (**A10**) was the most abundant anthocyanin (60.6% in A and 57.5% in LA), followed by cyanidin 3-*O*-galactoside (**A7**) (25.6% in A and 27.9% in LA). In blackthorn samples (B and LB), cyanidin-3-*O*-rutinoside (**A10**) was also

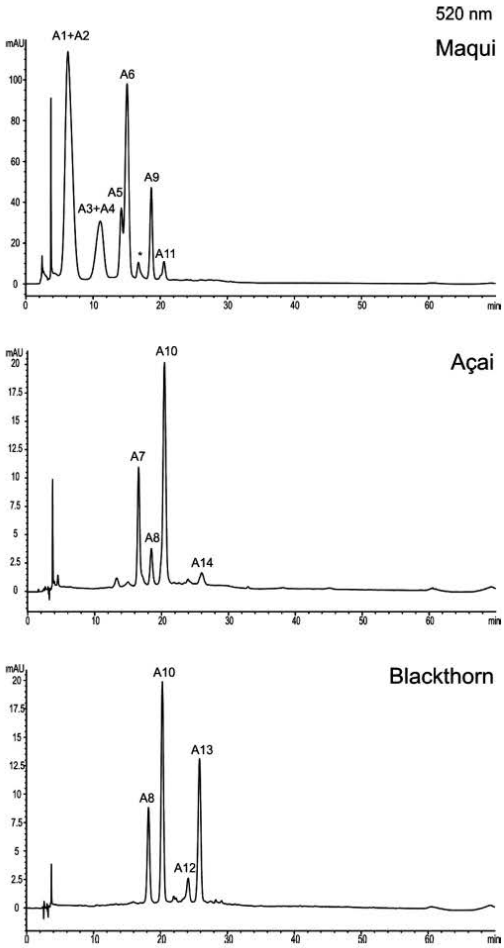

Figure 1. Chromatograms of berries registered at 520 nm. The identities of the compounds associated with the peaks shown here are given in Table 1.

the predominant anthocyanin (41.9% in B and 42.2% in LB), followed by peonidin 3-*O*-rutinoside (**A13**) (32.5% in B and 32.3% in LB).

Maqui samples (M, LM) also showed the highest contents of flavonols (quercetin and myricetin glycosides) (Table 3). Açaí and blackthorn samples displayed similar quantities of flavonols, quercetin 3-*O*-galactoside (**F5**) being the major flavonol in açaí (67.0% in A and 45% in LA), quercetin 3-*O*-rutinoside (**F4**) in LB (40.7%), and quercetin 3-*O*-xyloside (**F8**) in B (39.5%) (Table 3). With respect to hydroxycinnamic acid derivatives, blackthorn samples (B, LB) recorded noticeably higher contents, owing to a large peak of 3-*O*-caffeoylquinic acid (**C3**) (70.6% in B and 70.5% in LB) (Table 3). Therefore, the new blends analyzed improved the levels of hydroxycinnamic acid derivatives when compared to the controls (Table 3).

Only lemon juice and blends displayed flavones and flavanones (Table 3). Flavones were mainly represented by

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Table 1. Phenolic Compounds Identified in Berries: Maqui, Açaí, and Blackthorn

	t_R	$[M + H]^+/[M - H]^-$	MS^n	nm (max)	berries		
					maqui	açaí	blackthorn
Anthocyanins							
A1, delphinidin 3-*O*-sambubioside-5-*O*-glucoside	6.2	759	465, 597, 303	524	+	−	−
A2, delphinidin 3,5-*O*-diglucoside	6.9	627	465, 303	524	+	−	−
A3, cyanidin 3,5-*O*-diglucoside	11.2	611	449, 287	516	+	−	−
A4, cyanidin 3-*O*-sambubioside-5-*O*-glucoside	11.6	743	449, 581, 287	516	+	−	−
A5, delphinidin 3-*O*-sambubioside	14.2	597	303	524	+	−	−
A6, delphinidin 3-*O*-glucoside	15.1	465	303	524	+	−	−
A7, cyanidin 3-*O*-galactoside	16.4	449	287	520	−	+	−
A8, cyanidin 3-*O*-glucoside	18.3	449	287	520	−	+	+
A9, cyanidin 3-*O*-sambubioside	18.7	581	287	516	+	−	−
A10, cyanidin-3-*O*-rutinoside	20.3	595	287	516	−	+	+
A11, cyanidin 3-*O*-glucoside-5-*O*-rhamnoside	20.6	595	449, 287	517	+	−	−
A12, peonidin 3-*O*-glucoside	24.2	463	301	520	−	−	+
A13, peonidin 3-*O*-rutinoside	25.8	609	301	520	−	−	+
A14,malvidin 3-*O*-glucoside	25.9	493	331	520	−	+	−
isomer of cyanidin 3,5-*O*-diglucoside[a]	16.7	611	449, 287	524	+	−	−
Noncolored Flavonoids							
ellagic acid derivatives							
E1, granatin B	23.8	951	933, 301	275	+	−	−
E2, ellagic acid hexoside	35.8	463	301	255, 360	+	−	−
E3, ellagic acid rhamnoside	40.1	447	301	265, 360	+	−	−
flavonols							
F2, myricetin 3-*O*-galoylglucoside	26.9	631	479, 317	260, 355	+	−	−
F3, myricetin 3-*O*-galactoside	30.0	479	317	260, 355	+	−	−
F4, myricetin 3-*O*-glucoside	30.9	479	317	260, 355	+	−	−
F5, quercetin 3-*O*-rutinoside	38.9	609	301	260, 360	+	−	+
F6, quercetin 3-*O*-galactoside	39.4	463	301	260, 360	+	+	−
F7, quercetin 3-*O*-hexoside-5-*O*-pentoside	40.2	595	463, 301	260, 360	−	−	+
F8, quercetin 3-*O*-glucoside	41.8	463	301	260, 360	+	+	−
F9, quercetin 3-*O*-xyloside	47.1	433	301	260, 360	+	−	+
F10, quercetin 3-*O*-arabinoside	49.1	433	301	260, 360	+	+	−
F11, quercetin 3-*O*-rhamnoside	50.7	446	301	260, 360	+	+	−
hydroxycinnamic acid derivatives							
C1 caffeoyldihydrocaffeoylquinic acid (1)[a]	6.7	517	335	330	−	−	+
C2, caffeoyldihydrocaffeoylquinic acid (2)[a]	7.0	517	335	330	−	−	+
C3, 3-*O*-caffeoylquinic acid	8.9	353	191, 179	330	−	+	+
C4, 3-*O*-*p*-coumaroylquinic acid	13.5	337	163	320	−	−	+
C5, 4-*O*-caffeoylquinic acid	16.2	353	173	330	−	−	+
C6, 5-*O*-caffeoylquinic acid	16.5	353	191	330	+	+	−
C7, 3-*O*-feruloylquinic acid	16.7	367	193	330	−	−	+
C8, 5-*O*-*p*-coumaroylquinic acid	24.9	337	191	330	−	+	−

[a]Tentatively identified as two isomers of caffeoyldihydrocaffeoylquinic acid.

diosmetin 6,8-di-*C*-glucoside (**L2**), followed by diosmetin 7-*O*-rutinoside (**L4**) and apigenin 6,8-di-*C*-glucoside (**L1**). With respect to flavanones, eriodictyol 7-*O*-rutinoside (**L10**) and hesperetin 7-*O*-rutinoside (**L11**) were found in similar amounts. Ferulic acid (**C9**) was the predominant hydroxycinnamic acid, followed by sinapic acid (**C10**) and 5-*O*-caffeoylquinic acid (**C6**) in lemon samples (Table 3). This characteristic composition was previously observed.[35] Nevertheless, the concentration of flavanones is lower and the amount of flavones is higher than those in previously found in 'Fino' lemon.[34] It is important to emphasize that flavonoids in lemon are variable according to cultivar, season, growth conditions, or maturity stage.[26,34]

Antioxidant Capacity. *DPPH•.* The antioxidant activity was measured by different methods, including DPPH• scavenging. The IC_{50} was used to compare samples (Table 4). Concerning controls, maqui (M) presented the highest activity, which was increased in the LM blend. LA and LB blends were also more effective than the respective controls and lemon juice. This increased antiradical activity in the blends with respect to controls was probably due to the effect of the new matrix enriched in phytochemicals groups in the new mixtures. Nonetheless, we could not find any correlation between anthocyanins and DPPH•. This is a different result from that previously reported with anthocyanin-based fruit extracts[36] or fruits,[37] showing direct correlations between these variables. Then, the higher activity observed with maqui samples (M, LM) explained by their higher anthocyanins content was also supported by the DPPH• scavenging capacity of the maqui fruits[12] and of açaí extracts,[38] as reported for an anthocyanin-rich extract.[23] With respect to blackthorn, a good

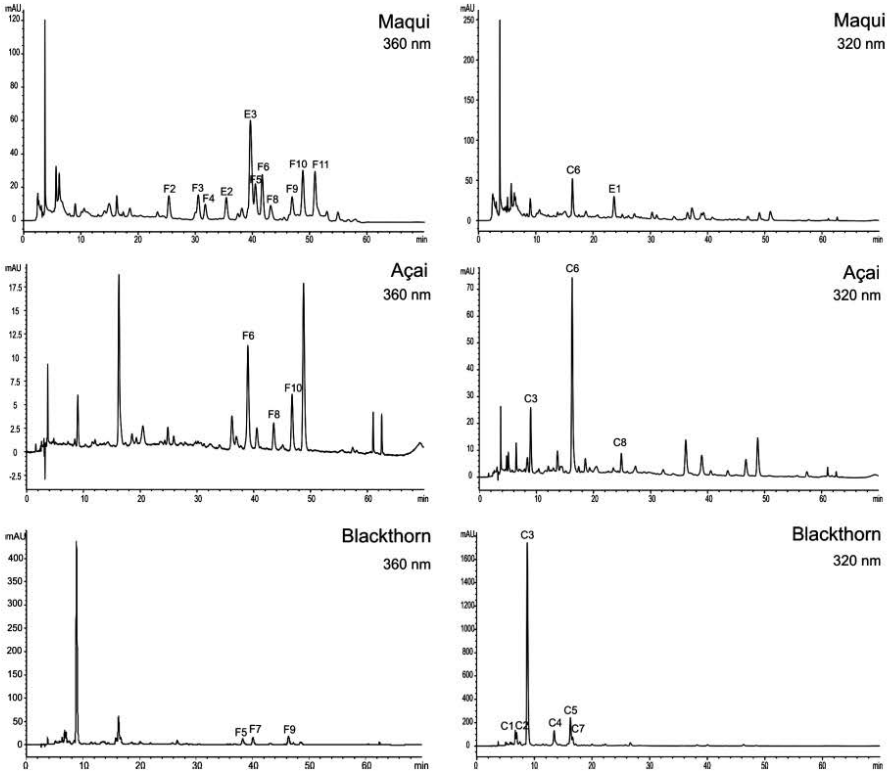

Figure 2. Chromatograms of berries recorded at 360 and 320 nm. The identities of the compounds associated with the peaks shown here are given in Table 1.

antioxidant activity against DPPH• was found in fresh juice and fresh fruits.[24,39]

Superoxide Radical. All samples led to low IC_{50} values in the superoxide ($O_2^{\bullet-}$) scavenging assay (Table 4), suggesting a high activity against this reactive oxygen species. The values ranged between 2.31 mg/mL (in LM) and 5.49 mg/mL (in B). The addition of blackthorn fruit to lemon juice (LB) resulted in the highest activity when compared to its control (B), whereas the opposite occurred for açaí (LA vs A). In previous studies on the results of $O_2^{\bullet-}$ the scavenging activity of flavonoids was associated not only with rutin, apigenin, neohesperidin, neoeriocitrin, and quercetin,[40] but also with anthocyanin-rich extracts,[41] even using freeze-dried açaí.[19] As far as we are aware, no previous studies about $O_2^{\bullet-}$ scavenging ability of maqui or blackthorn have been performed, and this result is useful for the biological evaluation of these berries. On the other hand, we found no significant correlation between $O_2^{\bullet-}$ scavenging activity or any fruit compounds, but that does not mean that other components of the matrix not detected or analyzed could have some activity.

Hypochlorous Acid. The lowest activity was observed against hypochlorous acid (HOCl). The IC_{50} values varied significantly between 15.28 mg/mL (in LM) and 42.80 mg/mL (in A) (Table 4). Lemon juice (L) exhibited higher activity than berry controls (M, A, or B), which was improved in the blends (Table 4). In addition, a significant correlation between flavones and flavanones and IC_{50} was found ($R = -0.869^*$ ($p < 0.05$) and $R = -0.928^{**}$ ($p < 0.005$), respectively). Previous works defined citrus pulp as an effective HOCl scavenger[42] and reported a direct correlation with total phenolics. This is consistent with the slightly higher effect of lemon juice in comparison with berry controls (M, A, or B). In addition, the increased antiradical activity of the new blends may be due by the addition of phytochemicals from the lemon and the compounds from berries, which can give more stability to lemon bioactive compounds, as previously reported.[1,2] These new data on HOCl scavenging ability of maqui, açaí, or blackthorn describe additional modes of action of these fruits in terms of antioxidant activity to support their potential use in food and health.

Hydroxyl Radical. Concerning the hydroxyl radical (•OH), samples were characterized by a reactively high antioxidant capacity (Table 4): IC_{50} values varied from 2.79 mg/mL (in LM) to 6.93 mg/mL (in L). Thus, among controls, blackthorn (B) displayed the best activity, and LM and LA were also very effective among the samples. The addition of blackthorn to lemon juice did not seem to affect the antioxidant capacity of the fruit, unlike what happened with the other blends. Anthocyanin-rich extracts from pomegranate (*Punica granatum* L.), and its three major anthocyanidins (delphinidin, cyanidin,

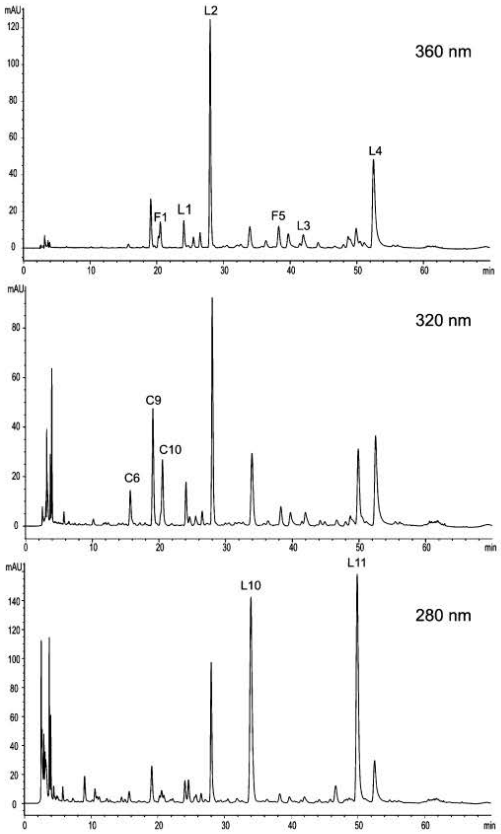

Figure 3. Chromatograms of lemon juice recorded at 360, 320 and 280 nm. The identities of the compounds associated with the peaks shown here are given in Table 2.

and pelargonidin),[43] and from rambutan (*Nephelium lappaceum* L.)[41] showed high activity against •OH, possibly by chelating Fe^{2+} rather than by direct scavenging of the radical. The poor •OH scavenging activity of purple açaí was also demonstrated before,[17] and no previous papers concerned the effect of maqui, blackthorn, or lemon juice. In this assay the effect of ascorbic acid has to be taken into consideration, because of its ability to redox cycle the metal ion required for hydroxyl radical generation, thus increasing the radical production.[29] No ascorbic acid was found in berries (data not shown), although lemon is rich in this nutrient.[26] Furthermore, no pro-oxidant effect was observed with lemon control and blends, probably owing to the phenolics contained in them.

When all of these data are taken into account, different assays can give a broader view to justification of the use of multiple antioxidant tests because most are radicals produced in human cells (superoxide radical, hypochlorous acid, and hydroxyl radical). It is interesting that although samples reacted differently against several reactive species, the novel beverage based on lemon juice and maqui berry (LM) was the most active in all assays (Table 4), as a result of its higher content in phytochemicals belonging to separate bioactive compound groups. The results suggested that phenolic compounds are not the only parameter selective for or indicative of the antioxidant potential; the quality composition and the interactions between compounds in the food matrix, such as the protection of lemon flavonoids by berry polyphenols, may be also involved.

AChE and BChE Inhibitory Activity. In recent years, the challenging search for natural inhibitors of cholinesterases involved in the etiology of a broad number of neurological disorders is increasing.[3] The IC_{50} values found for all of the tested controls and blends are presented in Table 4. All samples displayed AChE and BChE inhibitory activities. The results were similar for both enzymes, displaying a strong correlation between them (r = 0.993***, p < 0.001). Lemon juice (L) exhibited the best activity for both cholinesterases among control samples, showing also a direct correlation with its flavones and flavanones (R = −0.874* (p = 0.01) and R = −0.871* (p < 0.05) between total flavones and AChE and BChE, respectively; and R = −0.909** (p = 0.005) and R = −0.907** (p = 0.005) between total flavanones and AChE and BChE, respectively). The activity of lemon juice was not enhanced by maqui addition (LM), but significant increases were observed with blackthorn (LB) and açaí (LA), the latter being the most effective. Although this effect is considerably

Table 2. Phenolic Compounds Identified in Lemon Juice

compound	t_R	$[M - H]^-$	MS^n	nm (max)
flavones (360 nm)				
L1, apigenin 6,8-di-*C*-glucoside	24.1	593	503, 473	270, 345
L2, diosmetin 6,8-di-*C*-glucoside	28.0	623	503, 413, 383	270, 340
L3, 8-*C*-glucosylchrysoeriol	42.1	461	300	280, 350
L4, diosmetin 7-*O*-rutinoside	52.5	607	299	280, 345
flavonols (360 nm)				
F1, quercetin 3-*O*-rutinoside-7-*O*-glucoside	19.7	771	609, 301	265, 365
F5, quercetin 3-*O*-rutinoside	38.4	609	301	265, 365
hydroxycinnamic acid derivatives (320 nm)				
C6, 5-*O*-caffeoylquinic acid	16.5	353	191	330
C9, ferulic acid	19.1	175	169	330
C10, sinapic acid	20.6	205	189	330
flavanones (280 nm)				
L10, eriodictyol 7-*O*-rutinoside	34	595	287	280
L11, hesperetin 7-*O*-rutinoside	50	609	301	280

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Table 3. Quantification (millgrams per 100 mL) of Different Phenolic Compounds Present in Lemon Juice Control, Berry Controls, and Blends[a]

	controls				blends		
	lemon	maqui 5%	açaí 5%	blackthorn 5%	LM 5%	LA 5%	LB 5%
Anthocyanins							
A1, delphinidin 3-*O*-sambubioside-5-*O*-glucoside		16.66 ± 0.37[b]			16.52 ± 0.30[b]		
A2, delphinidin 3,5-*O*-diglucoside							
A3, cyanidin 3,5-*O*-diglucoside		4.29 ± 0.14[b]			4.53 ± 0.12[b]		
A4, cyanidin 3-*O*-sambubioside-5-*O*-glucoside							
A5, delphinidin 3-*O*-sambubioside		2.22 ± 0.01			2.40 ± 0.04		
A6, delphinidin 3-*O*-glucoside		7.41 ± 0.07			7.44 ± 0.13		
A7, cyanidin 3-*O*-galactoside			0.53 ± 0.01			0.54 ± 0.03	
A8, cyanidin 3-*O*-glucoside			0.18 ± 0.00	0.70 ± 0.09		0.17 ± 0.00	0.64 ± 0.01
A9, cyanidin 3-*O*-sambubioside		2.36 ± 0.03			2.49 ± 0.04		
A10, cyanidin-3-*O*-rutinoside			1.26 ± 0.02	1.52 ± 0.16	-	1.11 ± 0.06	1.42 ± 0.04
A11, cyanidin 3-*O*-glucoside-5-*O*-rhamnoside		0.52 ± 0.00			0.58 ± 0.01		
A12, peonidin 3-*O*-glucoside				0.24 ± 0.03			0.22 ± 0.00
A13, peonidin 3-*O*-rutinoside				1.18 ± 0.09			1.09 ± 0.04
A14, malvidin 3-*O*-glucoside			0.08 ± 0.00				
total anthocyanins		33.45 ± 0.61	2.09 ± 0.00	3.64 ± 0.37	34.00 ± 0.18	1.93 ± 0.10	3.36 ± 0.09
Noncolored Flavonoids							
flavones							
L1, apigenin 6,8-di-*C*-glucoside	0.90 ± 0.10				0.38 ± 0.01	0.76 ± 0.02	0.76 ± 0.02
L2, diosmetin 6,8-di-*C*-glucoside	7.02 ± 0.11				5.49 ± 0.04	5.50 ± 0.18	5.71 ± 0.20
L3, 8-*C*-glucosylchrysoeriol	nq				nq	nq	nq
L4, diosmetin 7-*O*-rutinoside	4.64 ± 1.64				2.82 ± 0.19	2.37 ± 0.03	2.39 ± 0.25
total flavones	12.55 ± 1.85				8.70 ± 0.22	8.63 ± 0.17	8.86 ± 0.02
ellagic acid derivatives							
E1, granatin B		6.35 ± 0.19			6.57 ± 0.83		
E2, ellagic acid hexoside		0.73 ± 0.07			0.55 ± 0.02		
E3, ellagic acid rhamnoside		3.73 ± 0.75			2.27 ± 0.19		
total ellagic acid derivatives		10.81 ± 0.63			9.40 ± 1.00		
flavonols							
F1, quercetin 3-*O*-rutinoside-7-*O*-glucoside	nq				nq	nq	nq
F2, myricetin 3-*O*-galoylglucoside		1.22 ± 0.14			0.71 ± 0.02		
F3, myricetin 3-*O*-galactoside		1.46 ± 0.19			1.17 ± 0.04		
F4, myricetin 3-*O*-glucoside		0.84 ± 0.10			0.45 ± 0.03		
F5, quercetin 3-*O*-rutinoside	0.97 ± 0.04	2.38 ± 0.24		1.11 ± 0.17	2.12 ± 0.04	0.81 ± 0.02	1.98 ± 0.04
F6, quercetin 3-*O*-galactoside		2.36 ± 0.37	2.31 ± 0.07		2.09 ± 0.08	1.68 ± 0.02	
F7, quercetin 3-*O*-hexoside-5-*O*-pentoside				1.21 ± 0.15			1.38 ± 0.05
F8, quercetin 3-*O*-glucoside		0.89 ± 0.11	0.50 ± 0.04		0.76 ± 0.05	0.54 ± 0.07	
F9, quercetin 3-*O*-xyloside		0.45 ± 0.02		1.52 ± 0.21	0.60 ± 0.01		1.51 ± 0.01
F10, quercetin 3-*O*-arabinoside		0.95 ± 0.15	0.63 ± 0.00		1.50 ± 0.15	0.71 ± 0.03	
F11, quercetin 3-*O*-rhamnoside		2.41 ± 0.37			1.49 ± 0.30		
total flavonols	0.97 ± 0.04	12.95 ± 1.69	3.45 ± 0.11	3.84 ± 0.55	10.87 ± 0.69	3.73 ± 0.13	4.88 ± 0.09
hydroxycinnamic acid derivatives (320 nm)							
C1, caffeoyldihydrocaffeoylquinic acid (1)				1.96 ± 0.25			1.83 ± 0.01
C2, caffeoyldihydrocaffeoylquinic acid (2)				1.78 ± 0.29			1.73 ± 0.01
C3, 3-*O*-caffeoylquinic acid			0.38 ± 0.03	31.26 ± 3.47		0.47 ± 0.01	33.58 ± 0.05
C4, 3-*O*-*p*-coumarylquinic acid				3.42 ± 0.45			3.15 ± 0.41
C5, 4-*O*-caffeoylquinic acid				4.42 ± 0.60			4.04 ± 0.14
C6, 5-*O*-caffeoylquinic acid	0.28 ± 0.00	1.07 ± 0.02	1.36 ± 0.19		1.58 ± 0.04	2.01 ± 0.02	0.45 ± 0.06
C7, 3-*O*-feruloylquinic acid				1.44 ± 0.17			1.65 ± 0.05
C8, 5-*O*-*p*-coumaroylquinic acid			0.13 ± 0.01			0.07 ± 0.01	
C9, ferulic acid	0.91 ± 0.00				1.09 ± 0.03	0.94 ± 0.01	1.03 ± 0.01
C10, sinapic acid	0.60 ± 0.01				0.87 ± 0.06	0.62 ± 0.01	0.64 ± 0.01

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Table 3. continued

	controls				blends		
	lemon	maqui 5%	açaí 5%	blackthorn 5%	LM 5%	LA 5%	LB 5%
Noncolored Flavonoids							
total cinnamic acid derivatives	1.79 ± 0.01	1.07 ± 0.02	2.25 ± 0.22	44.28 ± 1.77	3.54 ± 0.13	4.64 ± 0.03	47.64 ± 0.64
flavanones (280 nm)							
L10, eriodictyol 7-*O*-rutinoside	3.72 ± 0.09				3.28 ± 0.10	3.04 ± 0.08	3.12 ± 0.09
L11, hesperetin 7-*O*-rutinoside	3.90 ± 0.38				3.35 ± 0.09	3.33 ± 0.27	3.05 ± 0.11
total flavanones	7.63 ± 0.47				6.63 ± 0.24	6.37 ± 0.35	6.17 ± 0.20

[a]Values are the mean ± standard deviation (n = 3). L, lemon juice; M, 5% maqui in citric acid; A, 5% açaí in citric acid; B, 5% blackthorn in citric acid; LM, 5% maqui in lemon juice; LA, 5% açaí in lemon juice; LB, 5% blackthorn in lemon juice; nq, not quantified. [b]Anthocyanins A1 + A2 and A3 + A4 were quantified together.

Table 4. Antioxidant and Anticholinesterase (AChE and BChE) Activities of Control Fruits and Blends[a]

	IC_{50}					
	DPPH$^{\bullet}$	$O_2^{\bullet-}$	HOCl	$^{\bullet}$OH	AChE	BChE
L	13.27 ± 0.17 d	5.24 ± 0.43 e	25.71 ± 0.43 d	6.93 ± 0.22 d	13.18 ± 0.08 c	12.82 ± 0.22 c
M	9.06 ± 1.07 bc	3.10 ± 0.13 bc	40.49 ± 0.03 f	4.34 ± 0.19 c	21.76 ± 0.28 e	19.39 ± 0.36 d
A	18.91 ± 0.68 e	3.78 ± 0.24 cd	42.80 ± 0.29 g	4.72 ± 0.21 c	21.14 ± 0.38 e	19.87 ± 0.43 d
B	21.10 ± 0.58 f	5.49 ± 0.19 e	39.05 ± 0.63 e	3.30 ± 0.09 ab	19.51 ± 0.37 d	19.13 ± 0.44 d
LM	5.05 ± 0.14 a	2.31 ± 0.14 a	15.28 ± 0.06 a	2.79 ± 0.08 a	13.70 ± 0.13 c	13.38 ± 0.19 c
LA	7.39 ± 0.25 b	4.42 ± 0.18 d	18.84 ± 0.22 b	2.85 ± 0.42 a	8.83 ± 0.11 a	8.61 ± 0.14 a
LB	9.55 ± 0.83 c	2.96 ± 0.30 ab	21.90 ± 0.34 c	3.49 ± 0.06 b	10.44 ± 0.29 b	10.40 ± 0.26 b
LSD, $p < 0.05$	0.51	0.20	0.29	0.17	0.21	0.25

[a]Results are expressed in IC_{50} (n = 3, mg/mL). L, lemon juice; M, 5% maqui in citric acid; A, 5% açaí in citric acid; B, 5% blackthorn in citric acid; LM, 5% maqui in lemon juice; LA, 5% açaí in lemon juice; LB, 5% blackthorn in lemon juice. Means (n = 3) in the same columns followed by different letters are significantly different at $p < 0.05$ according to Tukey's test.

lower than that of pharmacological drugs used in neurological diseases, such as physotigmine[5] or galantamine,[44] this is a 100% natural food matrix with potential for daily consumption, without any side effects. Purified plant extracts also showed high activity, as in the case of methanolic extracts of *Lavanda viridis* (IC_{50} = 0.25 and 0.29 mg/mL for AChE and BuChE, respectively),[45] or the hydroxymethanolic extract of *Spergularia rubra* (IC_{25} = 3.68 and 4.29 mg/mL for AChE and BChE, respectively).[46] The cholinesterase inhibitory activity of natural products has been recently tested also in tomato seeds (IC_{20} = 2.4 mg/mL),[27] with slightly lower values than in our samples being reported. These results are of interest for developing natural food products for dietary intervention on cholinesterases using lemon juice, maqui, açaí, and blackthorn.

In this study new blends of lemon juice and different exotic (maqui, açaí) and Iberian (blackthorn) berries with health-promoting activities were developed, and their phytochemical profiles were described, revealing a wealth of bioactive phenolics: anthocyanins, flavonols, hydroxycinnamic acid derivatives, and ellagic acid derivatives in berries and flavones, flavanones, flavonols, and hydroxycinnamic acids in lemon juice. The results of the different radical scavenging methods indicated that the lemon−maqui novel beverage (LM) is the most interesting blend in terms of antioxidant activity. With respect to cholinesterases, all of the samples showed inhibitory activity: the highest potential was found with lemon juice among controls, lemon−açaí (LA) being the most promising blend. These results are of interest for developing natural AChE and BChE inhibitors demanded for health and nutrition, with potential interest in the design of new drinks with a nutritive-related function on cognitive aging-related health conditions as Alzheimer's disease, Parkinson's disease, or senile dementia among others.

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Notes

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3. PUBLICATION 5: DESIGN OF NEW BEVERAGES USING LEMON JUICE AND MAQUI BERRY STUDYING THE BIOACTIVE COMPOSITION AS WELL AS THEIR COMPOUNDS STABILITY, ANTIOXIDANT CAPACITY AND PHENOLIC CONTENT OVER 70 DAYS OF STORAGE PERIOD

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A novel beverage rich in antioxidant phenolics: Maqui berry (*Aristotelia chilensis*) and lemon juice

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ABSTRACT

In recent years, the interest in dietary antioxidants and bioactive compounds, mainly found in vegetables, has prompted research in the field of new polyphenol-rich drinks. The aim of the present work was to design new beverages using lemon juice and maqui (*Aristotelia chilensis*), rich in flavonoids and vitamin C. The composition of the new beverages as well as their compounds stability, antioxidant capacity and phenolic content over 70 days of storage period were studied. Results showed how anthocyanins and other phytochemicals from maqui preserved vitamin C and other flavonoids in the new mixtures owing to a higher rate of anthocyanin degradation. However, for the colour characteristics, the CIE*Lab* parameters displayed only slight variations, and the samples presented attractive colour during storage. The new beverages also had high values of *in vitro* antioxidant capacity, mainly owed to the maqui polyphenols, with a strong stability throughout study. Therefore, a new designed drink for the growing market of high nutritional and health-promoting food products has been developed.

1. Introduction

Nowadays, there is a growing dietary interest on health-promoting berries, which are fruits rich in anthocyanins among other phenolic phytochemicals, and other bioactive compounds (Seeram et al., 2008). Among berries, maqui (*Arisotelia chilensis* (Mol.) *Stuntz*) is a Chilean native evergreen shrub of the *Elaeocarpaceae* family that grows in Central and Southern areas of the country and produces red/purple colour berries about 6 mm in diameter. Fruits are usually eaten fresh or used for juice and jams (Escribano-Bailón, Alcalde-Eon, Muñoz, Rivas-Gonzalo, & Santos-Buelga, 2006). In the traditional native herbal medicine, infusions of maqui fruits and leaves have long been used to treat sore throats, kidney pain, digestive ailments (tumours and ulcers), fever, and scarring injuries (Suwalsky, Vargas, Avello, Villena, & Sotomayor, 2008). Recently, scientific research has demonstrated that these fruits have a strong *in vitro* antioxidant, anti-atherogenic and cardioprotective activities, and *in vitro* both adipogenesis and inflammation inhibitory effects, among others (Céspedes, El-Hafidi, Pavon, & Alarcon, 2008; Schreckinger, Wang, Yousef, Lila, & De Mejia, 2010a).

Therapeutical properties of maqui have been related to their high polyphenols content, concretely anthocyanins: delphinidin 3-sambubioside-5-glucoside, delphinidin 3,5-diglucoside, delphinidin 3-sambubioside, delphinidin 3-glucoside, cyanidin 3-sambubioside-5-glucoside, cyanidin 3,5-diglucoside, cyanidin 3-sambubioside, and cyanidin 3-glucoside (Escribano-Bailón et al., 2006; Schreckinger, Wang, et al., 2010a). Consequently, owing to the presence of these anthocyanins, maqui berries can also be used as natural colourants (Escribano-Bailón et al., 2006), giving an attractive red colour to new mixed-juices.

On the other hand, *Citrus* genus is the most important fruit crop in the world. Lemon (*Citrus limon* (L.) Burm. f.) is the third most important citrus crop (González-Molina, Domínguez-Perles, Moreno, & García-Viguera, 2010). Furthermore, lemon fruit is also a rich source of nutrients, including vitamin C (ascorbic acid + dehidroascorbic acid), minerals, citric acid, and flavonoids, which provide numerous health benefits (González-Molina et al., 2010). Vitamin C is probably the most important water-soluble antioxidant as well as an efficient scavenger of reactive oxygen species, and lemon is a rich source of this nutrient (González-Molina et al., 2010). With respect to flavonoids, hesperidin and eriocitrin (flavanones) and diosmetin glycosides (flavones) are the main compounds (Gil-Izquierdo, Riquelme, Porras, & Ferreres, 2004). Other notable flavonoids have been identified in lemon: vicenin-2 (flavone), diosmin (flavone), quercetin and myricetin (flavonols) as well as other hydroxycinnamic acids (Gil-Izquierdo et al., 2004;

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Hertog, Hollman, & Van de Putte, 1993). Taking into account the bioactive composition of lemon, a wide range of beneficial effects on the prevention of different kinds of cancer, cardiovascular diseases, glucose, lipid metabolism and obesity have been reported (Adibelli, Dilek, & Akpolat, 2009; Hertog et al., 1993).

Therefore, to supply antioxidants through diet it is of great interest polyphenol-rich beverages. The aim of this work was to produce new drinks using lemon juice and maqui berries at different concentrations (2.5% and 5% w/v), following the scope of previous reports directed towards the research of new beverages based on rich-in-antioxidants berries. Likewise, the phytochemical composition, antioxidant capacity, colour, and stability during storage at two different temperatures (4 °C and 25 °C) were studied to characterize these newly designed mixed-juices as novel, safe and acceptable drinks.

2. Materials and methods

2.1. Chemicals

Phenolic compounds were obtained commercially: cyanidin 3-glucoside (Polyphenols, Norway; >97% purity); hesperidin (Merck, Darmstadt, Germany; >90% purity); diosmin (Genay, France; >95% purity); gallic acid (Doesder. Chem. Co., Barcelona, Spain; >99% purity). Other reagents were, 2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt ($ABTS^{+}$) (Sigma, Steinheim, Germany); 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (Fluka Chemika, Neu-Ulm, Switzerland); Folin-Ciocalteu's Reagent (Sigma, Steinheim, Germany); sodium carbonate anhydre (Panreac Química S.A., Barcelona, Spain); potassium di-hydrogen phosphate (Panreac Química S.A., Barcelona, Spain); hexadecyltrimethylammonium bromide (Sigma, Steinheim, Germany); citric acid (Sigma, Steinheim, Germany); sodium benzoate (Panreac Quimica S.A., Barcelona, Spain); dimethylsulphoxide, formic acid, and methanol were all of analytical grade (Merck, Darmstadt, Germany); ascorbic acid (AA) and dehydroascorbic acid (DHAA), both from Sigma–Aldrich (Steinheim, Germany); 1,2-phenylenediamine dihydrochloride (OPDA) (Fluka Chemika, Neu-Ulm, Switzerland). Ultrapure water was produced using a Millipore water purification system (Molsheim, France).

2.2. Fruits

Commercial maqui berries were provided by 'Altalena' (Chile), freeze-dried and thawed at −20 °C until tests. Lemon juice was obtained from 'Fino' lemons freshly collected from CEBAS-CSIC's Experimental Farm ('La Matanza', Santomera, Murcia, SE Spain; 38°6′14″ N, 1°1′59″ W), using a domestic squeezer ('Citromatic', Braun Española S.A., Barcelona, Spain). Juice was stored frozen (−20 °C) until used.

2.3. Experimental design

Freeze-dried maqui berries were grinded and added to a volume of lemon juice to obtain final concentrations of 2.5% (w/v) and 5% (w/v) of the grind fruit in the beverage. In addition, control solutions in a 0.18 M citric acid buffer (pH 2.46) using the same proportions were prepared to study the behaviour of maqui phytochemicals without the lemon juice. Lemon juice was also assayed as control.

Homogenized mixtures and control solutions were centrifuged (5 min at 3500 rpm). After that, sodium benzoate (200 mg l^{-1}) was added in order to prevent spoilage. Mixtures and controls were stored in transparent glass vials (56 mm × 18 mm Ø; vol. 10 ml) with plastic screw-caps, and stored at both 4 °C and 25 °C in the dark for 70 days. Triplicate solutions were prepared for each experiment and all analytical measurements were done in triplicate. Analyses were carried out every 7 days for the first 28 days, and every 14 days during the rest of the experiment.

Samples were labelled as follows: L (lemon juice control), LM 2.5% (lemon juice plus 2.5% of maqui berry), LM 5% (lemon juice plus 5% of maqui berry), M 2.5% (2.5% maqui control solution in citric acid buffer), M 5% (5% maqui control solution in citric acid buffer).

2.4. pH, Titratable Acidity (TA), and Total Soluble Solids (TSS)

pH, Titratable Acidity (TA), and Total Soluble Solids (TSS) were evaluated as quality indexes following the method reported by Mena et al. (Mena et al., 2011). Results were expressed as g citric acid per 100 ml of sample in TA, and °Brix in TSS.

2.5. Analysis of phenolic compounds by RP-HPLC-DAD

All samples were centrifuged for 5 min at 10,500 rpm (model Sigma 1–13, B. Braun Biotech International, Osterode, Germany). Supernatant was filtered through a 0.45 μm PVDF filter (Millex HV13, Millipore, Bedford, Mass., USA) before injection into the HPLC system. For the identification and quantification of anthocyanins the method previously reported by González-Molina et al. (González-Molina, Moreno, & García-Viguera, 2009) was followed. The HPLC system was equipped with a Luna C_{18} column (25 cm × 0.46 cm i.d., 5 μm particle size; Phenomenex, Macclesfield, UK) with a C_{18} security guard (4.0 × 3.0 mm) cartridge system (Phenomenex, Macclesfield, UK), using as mobile phases 5% formic acid in water (v/v) (solvent A) and HPLC-grade methanol (solvent B) (Merck, Darmstadt, Germany). Elution was performed at a flow rate of 1 ml min^{-1} using a gradient starting with 1% B, reaching 20% B at 20 min, 40% B at 30, and 95% B at 35 and 39 min. Finally gradient came back at 1% B at 41 min until the end at 50 min. Chromatograms were recorded at 280, 360 and 520 nm. Different phenolics were characterised by chromatographic comparison with analytical standards and accordingly to previous reports (Schreckinger, Lotton, Lila, & de Mejia, 2010b; González-Molina et al., 2010) as well as quantified by the absorbance of their corresponding peaks. Flavanones were quantified as hesperidin at 280 nm; flavones as diosmin at 360 nm, and anthocyanins as cyanidin 3-glucoside at 520 nm.

2.6. Extraction and analysis of vitamin C

Vitamin C content were determined by HPLC as described by González-Molina et al. (González-Molina et al., 2010). AA and DHAA were identified and quantified by comparison with pattern areas from AA and DHAA. The vitamin C content was calculated by adding AA and DHAA content, and results were expressed as mg l^{-1}.

2.7. Colour measurements

Colour measurement was determined following the method reported by González-Molina et al. (González-Molina, Moreno, & García-Viguera, 2008a). Data (CIEL^*, a^* and b^*), were recorded and processed using the Minolta Software Chromacontrol S, PC-based colourimetric data system. *Hue angle* (H) was calculated from $\tan^{-1}(b^*/a^*)$ and Chroma (C^*) from $(a^{*2} + b^{*2})^{1/2}$.

2.8. Total phenolic content by the Folin–Ciocalteu's Reagent

Total phenolic content (TPC) was determined by the Folin–Ciocalteu's Reagent method adapted to a microscale according to a described procedure (Mena et al., 2011). Results were expressed as mg per 100 ml of gallic acid equivalents (GAE).

Table 1
pH, Titratable Acidity (TA) and Total Soluble Solids (TSS) during 70 days of storage.

Mixtures	pH	TA	TSS
L	2.12 ± 0.02a	5.53 ± 0.03b	7.20 ± 0.07c
M 2.5%	2.49 ± 0.05b	2.89 ± 0.08a	4.00 ± 0.08a
M 5%	2.52 ± 0.06b	2.95 ± 0.04a	5.00 ± 0.07b
LM 2.5%	2.14 ± 0.04a	5.84 ± 0.04c	8.40 ± 0.07d
LM 5%	2.17 ± 0.04a	6.03 ± 0.06d	9.20 ± 0.13e
LSD, $p < 0.05$	0.02	0.02	0.03

Values are mean ± standard deviation ($n = 18$).
TA (Titratable Acidity) is expressed as g citric acid per 100 ml juice.
TSS (Total Soluble Solids) is expressed as °Brix (25 °C).
Means (n=3) in the same columns followed by different letters are significantly different at $p < 0.05$ according to Tukey's test.

2.9. $ABTS^{+}$ assays of antioxidant capacity

All samples were centrifuged at 10,500 rpm (model EBA 21, Hettich Zentrifugen, Tuttlingen, Germany) during 5 min at room temperature. The free radical scavenging activity was determined using the $ABTS^{+}$ method in aqueous media according to Mena et al., (Mena et al., 2011). The antioxidant activity was evaluated by measuring the variation in absorbance at 414 nm after 50 min. Assays were measured by using 96-well micro plates (Nunc, Roskilde, Denmark) and Infinite® M200 micro plate reader (Tecan, Grödig, Austria). All reactions started by adding 2 µl of the corresponding diluted sample to the well containing the stock solution (250 µl). Final volume of the assay was 252 µl. Results were expressed as mM Trolox.

2.10. Statistical analyses

Data shown are mean values ($n = 3$). All data were subjected to analyses of variance (ANOVA) and a Multiple Range Test (Tukey's test), using PASW Statistics 18 software (Somers, New York, USA). Pearson correlation analysis was performed to corroborate relationships between selected parameters.

3. Results and discussion

3.1. Quality parameters

Concerning both pH and TA (Titratable Acidity) values, non-significant or relative differences were observed in the mixtures or the control juices for 70 days of storage, and also non-significant differences were found at the two studied temperatures; because of this, results were presented as average values for the whole experiment (Table 1). The pH of beverages with L juice (L, LM 2.5% and LM 5%) was lower than 2.2, whereas maqui berry controls (2.5% and 5%), were 2.49 and 2.52, respectively. In relation to TA, citric acid content in maqui control solutions was lower than in mixtures containing L juice, which presented normal values (around 6 g citric acid · 100 ml^{-1}) (González-Molina, Moreno, & García-Viguera, 2008b).

On the other hand, TSS content of both lemon and maqui control solutions were lower than those registered in the mixtures, as expected (Table 1). TSS values of all samples did not change considerably throughout the storage period at two temperatures considered.

3.2. Flavones and flavanones stability

Flavonoids concentration in lemon juice depends on the cultivar, and maturity stage, among other factors (González-Molina et al., 2008a). Flavanones and flavones were provided by lemon juice to the new mixtures, since these kind of phenolic compounds were not found in maqui controls. Main flavones quantified in lemon mixtures were vicenin-2 and diosmetin 6,8-diglucoside, whereas main flavanones were eriocitrin and hesperidin. Flavones initial values were similar in all beverages containing lemon juice:

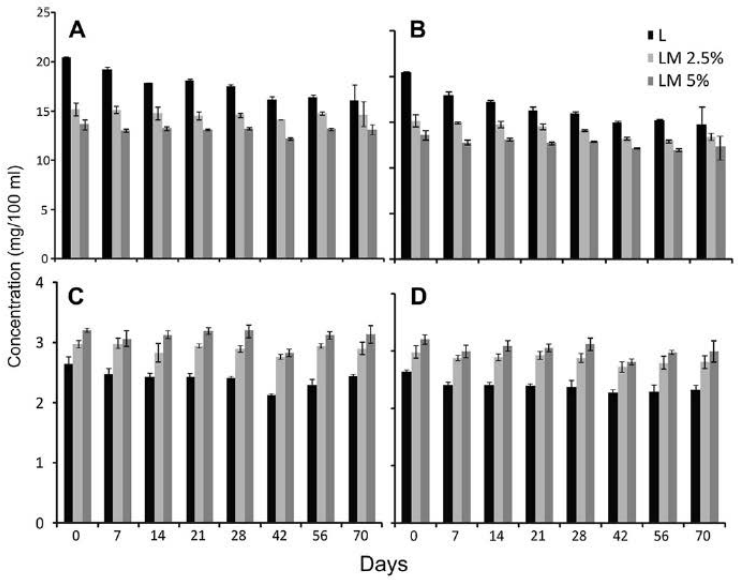

Fig. 1. Total lemon flavonoids at 4 °C (A), 25 °C (B) and diosmetin 6,8-diglucoside at 4 °C (C) and 25 °C (D) in 70 days of storage.

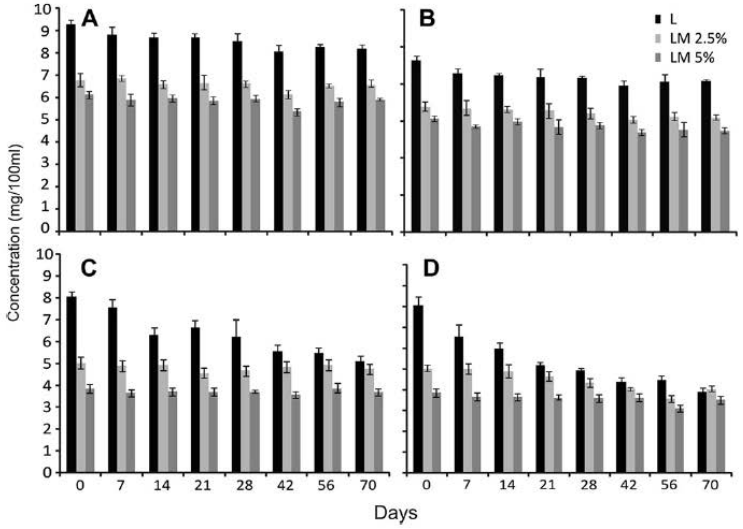

Fig. 2. Flavanones eriocitrin at 4 °C (A), 25 °C (B) and hesperidin at 4 °C (C) and 25 °C (D) in 70 days of storage.

0.44 and 2.95 mg 100 ml^{-1} for vicenin and diosmetin 6,8-diglucoside, respectively. In relation to flavanones, lemon juice showed the highest initial quantity for both eriocitrin and hesperidin (9.27 mg 100 ml^{-1} and 8.09 mg 100 ml^{-1}, respectively), whereas initial values of mixtures with maqui were lower in LM 2.5%: 6.78 mg 100 ml^{-1} and 5.05 mg 100 ml^{-1} for eriocitrin and hesperidin, respectively and in LM 5%: 6.14 mg 100 ml^{-1} and 3.86 mg 100 ml^{-1}.

Regarding the changes in concentration during the storage period, total lemon flavonoids (flavones plus flavanones) showed a decrease in concentration, stressed in the mixtures stored at 25 °C (Fig. 1). Lemon juice had the highest loss among all samples, 21.1% at 4 °C and 27.7% at 25 °C. On the contrary, maqui mixtures displayed a slight protection, as the fall in total lemon flavonoids was lower (Fig. 1). With respect to flavones, only small losses were observed in lemon juice control at 25 °C and they have been related to variations in diosmetin 6,8-diglucoside (12.1%), since vicenin-2 did not suffer almost any change (Fig. 2). Concerning flavanones, eriocitrin did not display significant losses, even though hesperidin was the most affected lemon flavonoid during storage (Fig. 2). Main decreases of hesperidin were registered in lemon juice control (L), reaching almost a 50% of the initial content by the end of the experiment. This fact could be attributed to the tendency to precipitate of flavanones as a consequence of their low solubility (Gil-Izquierdo et al., 2004). However, only 20.4 and 9.1% of the initial hesperidin was degradated in lemon-maqui mixtures (LM 2.5% and LM 5%, respectively) stored at 25 °C (Fig. 2). Thus, a possible stabilization effect of maqui berries on the hesperidin was observed, that had protected hesperidin of degradation, keeping lemon flavonoids content, in a dose-dependent manner, because with higher proportion of maqui, the hesperidin decreased. Similar observations, although in a lesser degree (5.6% and 4.3%, in LM 2.5% and 5%, respectively), have also been seen in samples stored at 4 °C.

3.3. Vitamin C content and changes during storage

Lemon juice is a natural source of vitamin C, as it was aforementioned, in contrast to maqui freeze-dried berries, where vitamin C was not detected. Consequently, vitamin C (calculated as the sum of AA and DHAA) was analysed only in those mixtures containing lemon juice (Fig. 3). Initially, the vitamin C content of

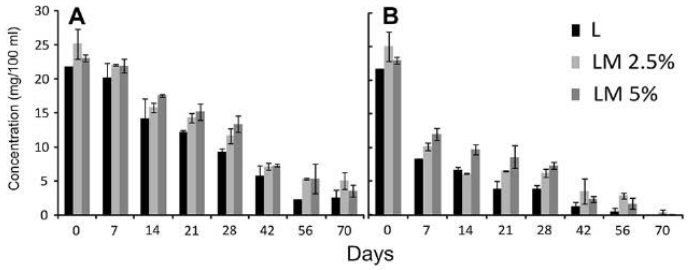

Fig. 3. Total vitamin C (AA + DHAA) for 70 days of storage at 4 °C (A) and 25 °C (B).

Table 2
Anthocyanins in maqui berries (*Aristotelia chilensis*) recorded at 520 nm.

Anthocyanins	Tr	nm (max)	M^+ (*m*/*z*)	MS^2 (*m*/*z*)
Delphinidin 3-sambubioside 5-glucoside	23.4	524	759	465, 597, 303
Delphinidin 3,5-diglucoside	24.7	524	627	465, 303
Cyanidin 3-sambubioside 5-glucoside	26.7	516	743	449, 581, 287
Cyanidin 3,5-diglucoside	26.7	516	611	449, 287
Delphinidin 3-sambubioside	28.7	524	597	303
Delphinidin 3-glucoside + unidentified acylated anthocyanin	29.5	524	465	303
Tentatively identified as isomer of 3b	30.4	516	611	449, 287
Cyanidin 3-sambubioside	31.1	516	581	287

lemon control (L) and mixtures (LM 2.5% and 5%) was similar, ~25 mg 100 ml^{-1}.

During the storage period, a significant fall in vitamin C content over first days was seen, being more striking in those samples stored at 25 °C. Concretely, vitamin C of lemon juice control (L) decreased 68% during the first 14 days of storage at 25 °C, while at 4 °C, the losses were only of a 35% (Fig. 3). However, the rate of decrease in vitamin C content was lower in the mixtures with maqui, with retention of this nutrient until the end of the experiment. Like this, LM 5% was the best mixture protecting vitamin C during the first 14 days, showing a degradation rate of 24% and 57% at 4 °C and 25 °C, respectively (Fig. 3). The LM 2.5% mixtures did not offer such protection with similar levels to the lemon juice control (L) (Fig. 3). This protective effect of berries on vitamin C has already been reported in models of beverages of lemon juice enriched with berry concentrates by González-Molina et al. (González-Molina et al., 2008b).

3.4. Anthocyanins

Anthocyanins of *A. chilensis* (Table 2) have been identified previously by Escribano-Bailón et al., and Schreckinger et al., (Escribano-Bailón et al., 2006; Schreckinger, Lotton, et al., 2010b). Seven of them plus one isomer were confirmed by HPLC-DAD-ESI-MS^n analysis. Initial values of each anthocyanin were similar in all samples with the same percentage of maqui berries powder, as well as in the respective total content (Table 3).

The total content of anthocyanins tended to decrease in all samples tested at 25 °C (Fig. 4), whereas the losses were of less intensity at 4 °C, condition that preserved anthocyanins during storage. It is remarkable that the anthocyanin degradation rate was clearly influenced by the presence of lemon juice in both studied temperatures. In fact, any loss of anthocyanins in maqui controls (M 2.5% and M 5%) was recorded at 4 °C; nevertheless, anthocyanins content of mixtures with lemon juice decreased by 19% and 11% for LM 2.5% and LM 5%, respectively, by the end of the experiment. The same results were achievable in a more emphasized way at 25 °C, as the fall in anthocyanins for both M 2.5% and M 5% was about 37%, while it was 74% and 56% for LM 2.5% and LM 5%, respectively. This effect can be attributed to: 1) the mutual degradation of anthocyanins and ascorbic acid (AA) in the presence of oxygen (Sondheimer & Kertesz, 1953), probably by a free radical mechanism (Iacobucci & Sweeny, 1983); and 2) the degradation products of AA (DHAA, H_2O_2, and furfurals, among others) that can also led to the breakdown of anthocyanins (Özkan, 2002).

Likewise, it is worth mentioning that the mixture with lower maqui concentration (LM 2.5%) was more affected with regard to the total anthocyanins than that with higher maqui dose (LM 5%) at the two studied temperatures.

3.5. Anthocyanins vs. vitamin C

Mutual degradation of both anthocyanins and ascorbic acid has been broadly reported (García-Viguera & Bridle, 1999; Özkan, 2002). Nonetheless, as it was aforementioned, whereas degradation rates of vitamin C were lower in lemon mixtures than in lemon controls, the anthocyanins from mixtures showed higher degradation than in the maqui controls. Therefore, a protective effect of anthocyanins on vitamin C has been exhibited. In this way, other authors have recorded the same protective effect in different models or beverages: Poei-Langston and Wrolstad (Poei-Langston & Wrolstad, 1981), and Bordignon-Luiz et al. (Bordignon-Luiz, Gauche, Gris, & Falcão, 2007), suggesting the protective effect of flavonols on ascorbic acid in an "ascorbic acid-anthocyanin-flavonol" model system. Iversen (Iversen, 1999), found that the degradation rate of anthocyanins was 3–4 times faster than the ascorbic acid in blackcurrant nectar, and Kaack and Austed (Kaack & Austed, 1998), remarked a protective effect on ascorbic acid when flavonols and anthocyanins were both present simultaneously. Hence, regardless implicated processes remain still unknown, it is a point worth mentioning the reduction of vitamin C degradation rate as a likely consequence of the combination of lemon juice phytochemicals with other bioactive compounds from the maqui berries.

3.6. Colour changes during storage

In general, colour parameters were similar among samples with the same concentration of maqui berry, even over time. Lightness ($CIEL^*$ value) tended to increase among all the samples and for both temperatures, being more stressed in those samples stored at 25 °C. Likewise, despite statistical significant differences recorded for samples stored at 4 °C, changes were not too relevant, except for L, probably due to flavanones precipitation (Gil-Izquierdo et al., 2004) (Tables 4 and 5).

Table 3
Initial values of anthocyanins content from maqui berry of the different mixtures studied.

Anthocyanins from maqui	M 2.5%	M 5%	LM 2.5%	LM 5%
Dp 3-smb-5-glc	38.84 ± 2.49d	75.55 ± 0.06f	38.15 ± 1.15c	72.94 ± 4.04c
Dp 3,5-diglc	37.73 ± 1.91cd	71.45 ± 0.54e	38.20 ± 1.63c	73.52 ± 3.70c
Cy 3,5-digly	15.53 ± 1.11ab	30.02 ± 0.04b	15.80 ± 0.82ab	29.82 ± 2.23ab
Dp 3-smb	18.61 ± 0.99b	35.75 ± 0.73c	19.17 ± 0.71b	36.77 ± 1.78b
Dp 3-glc	34.41 ± 1.75c	61.51 ± 1.48d	39.51 ± 1.88c	75.36 ± 3.15c
Cy 3-smb	12.78 ± 1.15a	23.96 ± 0.66a	13.82 ± 0.65a	26.90 ± 1.76a
LSD, $p < 0.05$	1.35	0.62	1.01	2.38
TAnt	**157.87 ± 8.60**	**300.34 ± 3.36**	**165.86 ± 8.60**	**317.45 ± 16.83**

Dp 3-smb-5-glc, (Delphinidin 3-sambubioside-5-glucoside); Dp 3,5-diglc (Delphinidin 3,5-diglucoside); Cy 3,5-digly, (Cyanidin 3,5-diglucoside + Cyanidin 3-sambubioside-5-glucoside); Dp 3-smb (Delphinidin 3-sambubioside); Dp 3-glc (Delphinidn 3-glucoside); Cy 3-smb, (Cyanidin 3-sambubioside).
Values are mean ± standard deviation ($n = 3$) expressed as mg·100 ml^{-1} juice.
Means (n=3) in the same columns followed by different letters are significantly different at $p < 0.05$ according to Tukey's test.

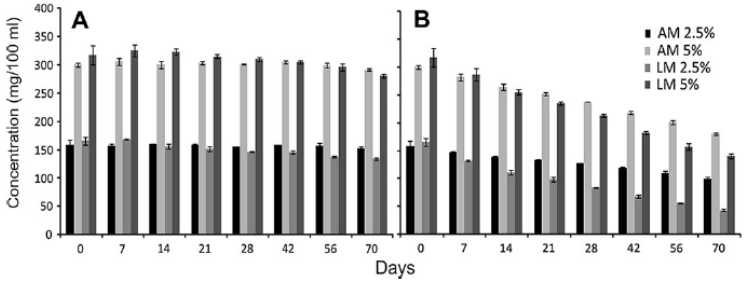

Fig. 4. Total anthocyanins content from maqui-samples during storage at 4 °C (A) and 25 °C (B).

Taking into account both redness (CIE$L a^*$) and yellowness (CIE$L b^*$), a similar trend was found among them for all the samples (a strong correlation between these parameters was recorded: $R^2 \sim 0.99$; $p < 0.001$). While a decrease in L was detected, an augment for all the other samples, both mixtures and controls, occurred. Likewise, variations were higher in those samples stored at 25 °C and, in spite of the statistically significant differences that there were not so considerable.

Table 4
Stability of CIE$L^*a^*b^*$ values in samples during storage at 4 °C.

4 °C	Days	L	M 2.5%	M 5%	LM 2.5%	LM 5%
CIEL^*	0	75.26a	29.06a	15.47a	24.82a	15.02a
	7	82.98b	31.27b	16.62b	25.32ab	15.04a
	14	83.57b	31.68b	17.10bc	26.04bc	15.76ab
	21	88.74c	32.37b	17.78d	26.96d	16.14b
	28	89.27c	31.72b	17.33cd	26.55cd	15.62ab
	42	89.46c	31.76b	17.18bcd	29.08e	16.21b
	56	89.39c	30.99b	16.79bc	31.22f	16.28b
	70	89.90c	32.16b	17.75d	32.41g	17.27c
	LSD	0.55***	0.44***	0.18***	0.25***	0.25***
CIEa^*	0	2.35e	60.42a	47.92a	57.29a	47.83a
	7	1.20d	62.32b	49.46b	57.71ab	47.96ab
	14	0.90c	62.77bc	50.08bc	58.31bc	48.86c
	21	0.28b	63.23c	50.89c	59.20d	49.35c
	28	0.02ab	62.74bc	50.37bc	58.94cd	48.74bc
	42	−0.02a	62.61bc	50.14bc	61.16e	49.36c
	56	0.03ab	62.24b	49.75b	63.41f	49.50c
	70	−0.01a	62.99bc	50.84c	63.99f	50.55d
	LSD	0.08***	0.26***	0.28***	0.23***	0.23***
CIEb^*	0	12.47d	49.95a	26.52a	42.63a	25.75a
	7	8.17c	53.77b	28.50ab	43.50ab	25.78a
	14	7.37c	54.46b	29.32bc	44.73bc	27.01ab
	21	4.01ab	55.64b	30.49c	46.32d	27.68b
	28	4.36b	54.55b	29.72bc	45.62cd	26.77ab
	42	3.36a	54.61b	29.47bc	49.99e	27.79b
	56	4.58b	53.29b	28.79abc	53.67f	27.91b
	70	4.32ab	55.30b	30.45c	55.72g	29.63c
	LSD	0.28***	0.77***	0.53***	0.44***	0.43***
Chroma	0	12.69d	78.38a	54.77a	71.41a	54.16a
	7	8.26c	82.31b	57.09b	72.25ab	54.45ab
	14	7.42c	83.10b	58.04bc	73.50bc	55.83bc
	21	4.02ab	84.23b	59.32c	75.17d	56.58c
	28	4.36ab	83.13b	58.48bc	74.56cd	55.61abc
	42	3.36a	83.08b	58.16bc	78.81e	56.65c
	56	4.58b	81.93b	57.48b	83.00f	56.83c
	70	4.32ab	83.84b	59.26c	84.85g	58.59d
	LSD	0.29***	0.70**	0.49***	0.41***	0.43***
Hue angle	0	79.34a	39.57a	28.96a	36.65a	28.28a
	7	81.68b	40.79b	29.95ab	37.00ab	28.26a
	14	83.08b	40.95b	30.34ab	37.50bc	28.94ab
	21	85.97c	41.35b	30.93b	38.04d	29.29b
	28	89.73d	41.00b	30.55ab	37.74cd	28.78ab
	42	90.37d	41.09b	30.44ab	39.26e	29.38b
	56	89.69d	40.57ab	30.05ab	40.29f	29.41bc
	70	90.14d	41.26b	30.92b	41.06g	30.37c
	LSD	0.54***	0.30**	0.34**	0.15***	0.28***

Means (n=3) in the same columns followed by different letters are significantly different at $p < 0.05$ according to Tukey's test. LSD, $p < 0.01$ (**), $p < 0.001$ (***).

Table 5
Stability of CIE$L^*a^*b^*$ values in samples during storage at 25 °C.

25 °C	Days	L	M 2.5%	M 5%	LM 2.5%	LM 5%
CIEL^*	0	75.26a	29.06a	15.48a	24.82a	15.02a
	7	77.46ab	32.39b	17.87b	28.29b	17.00b
	14	79.39b	33.29bc	19.47c	30.83c	18.79c
	21	87.88c	34.65cde	20.32d	33.59d	20.07d
	28	88.37cd	34.18cd	20.64d	34.02d	19.92d
	42	90.23cd	35.76ef	22.72e	36.42e	21.98e
	56	90.62d	35.21de	22.67e	36.98e	22.57ef
	70	90.86d	36.94f	24.01f	39.30f	23.69f
	LSD	0.77***	0.44***	0.24***	0.46***	0.42***
CIEa^*	0	2.35d	60.42a	47.92a	57.29a	47.83a
	7	2.05d	63.02b	50.91b	59.53b	50.08b
	14	1.41c	63.70bc	52.86c	61.54c	52.28c
	21	0.27b	64.42cd	53.75cd	63.92d	53.71c
	28	−0.04ab	63.81bcd	54.02d	63.91d	53.55c
	42	−0.17ab	64.49cd	56.00e	64.39d	55.55d
	56	−0.21a	64.47cd	56.04e	64.59d	56.26d
	70	−0.27a	64.91d	57.06f	64.34d	57.02d
	LSD	0.15***	0.32***	0.30***	0.25***	0.47***
CIEb^*	0	12.47d	49.95a	26.52a	42.63a	25.75a
	7	11.83cd	55.69b	30.66b	48.72b	29.16b
	14	10.88c	57.24bc	33.41c	53.00c	32.24c
	21	5.92b	59.59cde	34.88d	57.76d	34.44c
	28	5.88b	58.78cd	35.43d	58.49d	34.20c
	42	3.62a	61.50ef	39.02e	62.62e	37.91d
	56	4.75ab	60.54de	38.94e	63.61e	38.75d
	70	4.40a	63.42f	41.24f	67.60f	40.68e
	LSD	0.36***	0.76***	0.42**	0.76***	0.75***
Chroma	0	12.69d	78.38a	54.77a	71.41a	54.16a
	7	12.01cd	84.10b	59.43b	76.86b	57.95b
	14	10.97c	85.64bc	62.53c	81.22c	61.42c
	21	5.93b	87.71cd	64.08d	86.13d	63.81c
	28	5.88b	86.76cd	64.60d	86.63d	63.54c
	42	3.60a	89.11de	68.25e	89.82e	67.16d
	56	4.75ab	88.44de	68.24e	90.65e	68.32de
	70	4.42a	90.75e	70.41f	93.34f	70.05e
	LSD	0.41***	0.73***	0.47**	0.65***	0.79***
Hue angle	0	79.34a	39.57a	28.96a	36.65a	28.28a
	7	80.18ab	41.47b	31.04b	39.23b	30.11b
	14	82.62b	41.95bc	32.30c	40.73c	31.66c
	21	87.49c	42.78cde	32.98d	42.12d	32.67c
	28	90.36d	42.65cd	33.26d	42.47d	32.55c
	42	92.66de	43.64ef	34.86e	44.20e	34.19d
	56	92.51de	43.20de	34.79e	44.56e	34.56d
	70	93.59e	44.34f	35.85f	46.41f	35.50d
	LSD	0.86***	0.27***	0.19***	0.33***	0.41***

Means (n=3) in the same columns followed by different letters are significantly different at $p < 0.05$ according to Tukey's test. LSD, $p < 0.01$ (**), $p < 0.001$ (***).

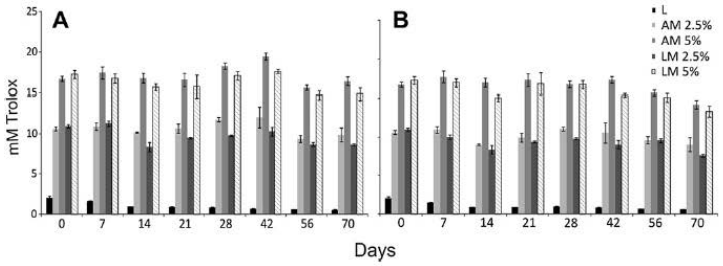

Fig. 5. Antioxidant capacity (mM Trolox) reported by $ABTS^+$ method for 70 days of storage at 4 °C (A) and 25 °C (B).

With respect to *Chroma* and *Hue* angle, an increase was noted for all the samples at all temperatures, apart from a fall in *Chroma* values of L, as it was expected considering the strong correlation existing between CIEa^* and CIEb^*.

On the other hand, regardless that losses in total anthocyanin contents over time were found, the red colouration remained quite stable, until the end of the study, as a result of the formation of other coloured-polymers (Boulton, 2001), or copigmentation between anthocyanins and flavonols that can also modify the colour expression by increasing *Chroma* along with a tone shift towards orange tonalities over time (González-Manzano, Dueñas, Rivas-Gonzalo, Escribano-Bailón, & Santos-Buelga, 2009). The same effect has recently been reported by González-Molina for elderberry concentrate mixed with lemon juice (Unpublished data), unlike in other red fruits (pomegranate, aronia, and grapes) this effect has been pointed out in opposite way (González-Molina et al., 2008b, 2009).

3.7. *In vitro antioxidant activity ($ABTS^+$ assay)*

Concerning the total *in vitro* antioxidant capacity measured by the $ABTS^+$ method, Lemon juice controls were lower (2.03 ± 0.21 mM Trolox) than the maqui controls (10.61 ± 0.25 and 16.69 ± 0.33 mM Trolox for M 2.5% and M 5%, respectively). Likewise, the new beverages reported similar levels than red-controls ones (10.94 ± 0.24 and 17.25 ± 0.46 for LM 2.5% and 5%, respectively), supporting the strong *in vitro* antioxidant capacity of maqui, attributed to their polyphenolic content (Céspedes, Alarcon, Avila, & Nieto, 2010; Céspedes et al., 2008; Miranda-Rottmann et al., 2002).

Regarding changes over the storage period, there were not remarkable differences between the two studied temperatures (Fig. 5). The L controls displayed a decrease of antioxidant capacity by about 70% at the end of the study, correlated to vitamin C degradation ($R^2 = 0.933$, $p < 0.01$ in L 4 °C; and $R^2 = 0.956$, $p < 0.001$ in L 25 °C). Nevertheless, when new mixtures (LM 2.5 and 5%) were analysed, the antioxidant capacity was rather constant and did not exceed 20% losses at 4 °C and 30% at 25 °C. This protective effect was probably due to the protection of vitamin C already discussed.

3.8. *Total phenolics*

The total phenolics content (TPC) analysed by the Folin–Ciocalteu's Reagent method was 62.97 ± 1.83, 143.07 ± 5.53, and 243.64 ± 8.08 mg GAE·100 ml^{-1} for L, M 2.5%, and M 5% controls, respectively. Moreover, when TPC of the new mixtures was measured, LM 2.5% and LM 5% showed the additive TPC of their components (187.80 ± 5.41 and 279.84 ± 5.15 mg gallic acid·100 ml^{-1}, respectively). Likewise, phenolic content determined by FCR method reported considerably higher concentrations than those determined by HPLC methods (Fig. 6). The results obtained by this method are not suitable to the total phenolics determination because the reagent react not only with phenolics but also with a variety of non-phenolic reducing compounds including tertiary aliphatic amines, tertiary amine-containing biological buffers, amino acids (tryptophan), hydroxylamine, hydrazine, certain purines, and other organic and inorganic reducing agents leading to overvaluation of the total phenolics content (Ikawa, Schaper, Dollard, & Sasner, 2003). Furthermore, different phenolics can present different answers with the Folin–Ciocalteu's Reagent, presenting lower absorption which it leads to a underestimation of various compounds (Vinson, Su, Zubik, & Bose, 2002).

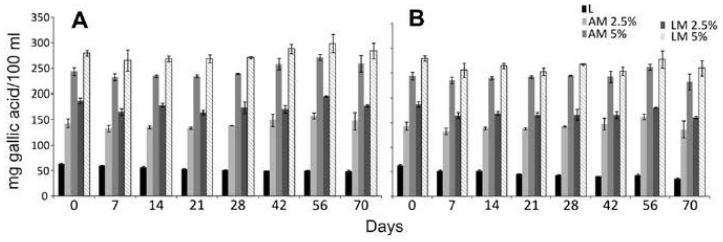

Fig. 6. Total phenolic content (TPC) measured for 70 days at 4 °C (A) and 25 °C (B) by Folin–Ciocalteu's method.

Concerning the evolution of juices over time, the TPC remained quite stable for all the beverages, recording only significant losses for L controls (22% at 4 °C and 44% at 25 °C), due in some extent to the possible precipitation of flavanones (Gil-Izquierdo et al., 2004; González-Molina et al., 2008b) (Fig. 6). Curiously, in maqui controls increased their TPC over time, which could be associated with the formation of secondary metabolites able to react with Folin–Ciocalteu's Reagent.

4. Conclusions

The novel new designed beverages of maqui berries and lemon juice have shown protective interactions among bioactive phytochemicals and a good stability over time with respect to the analytical parameters studied. The vitamin C of lemon juice was preserved in those mixtures containing maqui, thanks mainly to the anthocyanins from these berries. Likewise, hesperidin and, hence, lemon flavonoids, were protected by maqui as well. On the contrary, anthocyanins from new mixed-drinks suffered a severe decrease by the presence of lemon juice. CIE*Lab* parameters were generally stable, showing the new beverages a powerful and attractive red colour throughout the study. Finally, initial high levels of antioxidant capacity and total phenolics content from the mixtures remained quite stable over time, except for the lemon juice. In summary, new drinks rich in bioactive phytochemicals, had a high *in vitro* antioxidant activity as well as an attractive colour well preserved throughout the study period, especially at 4 °C. Further approaches in the evaluation of their bioavailability and biological activity are necessary to verify their potential *in vivo* beneficial effects for nutrition and health.

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4. PUBLICATIONS 6 AND 7: DESIGN OF NEW ISOTONIC BEVERAGES, ENRICHED WITH POLYPHENOLS FROM LEMON JUICE AND BERRIES, COMPARING TO COMMERCIALLY-AVAILABLE ISOTONIC DRINKS AND EVALUATING THE ORGANOLEPTIC, PHYTOCHEMICAL AND BIOLOGICAL CHARACTERISTICS OVER A STORAGE PERIOD OF 70 DAYS.

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RESEARCH ARTICLE

New isotonic drinks with antioxidant and biological capacities from berries (maqui, açaí and blackthorn) and lemon juice

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Abstract

The aim of the study was to design new isotonic drinks with lemon juice and berries: maqui [*Aristotelia chilensis* (Molina) Stuntz], açaí (*Euterpe oleracea* Mart.) and blackthorn (*Prunus spinosa* L.), following on from previous research. Quality parameters – including colour (CIELab parameters), minerals, phytochemical identification and quantification by high-performance liquid chromatography with diode array detector, total phenolic content by the Folin–Ciocalteu reagent, the antioxidant capacity ($ABTS^{\cdot+}$, DPPH• and $O_2^{\cdot-}$ assays) and biological activities (*in vitro* alpha-glucosidase and lipase inhibitory effects) – were tested in the samples and compared to commercially available isotonic drinks. The new isotonic blends with lemon and anthocyanins-rich berries showed an attractive colour, especially in maqui samples, which is essential for consumer acceptance. Significantly higher antioxidant and biological effects were determined in the new blends, in comparison with the commercial isotonic beverages.

Keywords

Berries, bioactives, citrus, diabetes, isotonic drinks, obesity

History

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Introduction

Health nutrition and wellness are growing areas of interest in the global markets of new developments in beverages, where juices, sport drinks and vitamin waters have an inherently healthy image, being formulated for specific benefits – beyond hydration – with regard to sports and energy boosts. The functional beverage market has increased steadily over the past decade, with a sharper rise in the last couple of years. According to Datamonitor, the global non-alcoholic beverage market is valued at just under $500 billion worldwide, with Europe accounting for a large portion: $189 billion (Fortitech, 2012). Isotonic drinks – thirst-quenching beverages used in sports and related activities, to rehydrate, boost energy and replenish electrolytes lost to sweating – contain simple carbohydrates, minerals, electrolytes (e.g. sodium, potassium, calcium, magnesium) and sometimes vitamins or additional nutrients (Segen, 1992). Worldwide, the fortified sports drink market topped nearly $18 billion in 2008 (Fortitech, 2012). It has been suggested recently that acute and strenuous exercise can increase oxidative stress through the enhanced formation of reactive oxygen species (ROS) and nitrogen species (Medina et al., 2012). Hence, a new functional and isotonic beverage with natural antioxidants from berries and lemon juice may contribute to avoidance of the negative redox balance generated by intense sporting activity. Recent research has highlighted the increased antioxidant capacity of lemon juice-based beverages (Gironés-Vilaplana et al., 2012a,b; González-Molina et al., 2012).

The fruits selected for designing the beverages were chosen according to their known bioactivity and composition. Lemon juice was chosen as it is rich in vitamin C, minerals, citric acid and bioactive flavonoids, which can provide health benefits beyond nutrition regarding cardiovascular disease, cancer, diabetes and obesity (González-Molina et al., 2010). Maqui, a common edible berry from central and southern Chile, is a source of natural colourants due to the presence of anthocyanins. Various reports have linked the phenolics of maqui with its high antioxidant capacity (Rubilar et al., 2011), *in vitro* inhibition of adipogenesis and inflammation (Schreckinger et al., 2010), cardioprotection (Céspedes et al., 2008) and *in vitro* and *in vivo* anti-diabetic effects (Rojo et al., 2011; Rubilar et al., 2011). Açaí is a palm tree berry from the Amazon River area in South America. Potential benefits have been attributed to açaí fruits, extracts and juices: antioxidant and anti-inflammatory activity (Schauss et al., 2006), protection of brain cells (Poulose et al., 2012), prevention of endothelial dysfunction in hypertension (da Costa et al., 2012), reduction of selected markers of metabolic disease risk (Udani et al., 2011), pain reduction and improved mobility (Jensen et al., 2011) and inhibition of urinary bladder carcinogenesis (Fragoso et al., 2012). Blackthorn is a fruit of deciduous shrubs native to Europe, mainly Spain, Portugal and Turkey (Barros et al., 2010). It is commonly used in the manufacture of jams or is macerated with aniseed liqueur to obtain a digestive alcoholic drink called patxarán. Blackthorn is also cited as an astringent, diuretic and purgative (Barros et al., 2010) and has recently been shown to possess antioxidant properties (Ganhão et al., 2010). Thus, the aims of this work were to design new isotonic beverages, based on lemon juice and berry fruits, and to carry out evaluation of their quality and

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biological activity (antioxidant capacity and effects on lipid and glucose metabolism enzymes), with regard to their potential use as novel functional ingredients and products.

Materials and methods

Chemicals

The 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$), 2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)diammonium salt (ABTS^{+}), Folin–Ciocalteu reagent, β-nicotinamide adenine dinucleotide (NADH), phenazine methosulphate (PMS), nitrotetrazolium blue chloride (NBT), trizina hydrochloride, 4-nitrophenyl alpha-*D*-glucopyranoside, alpha-glucosidase from *Saccharomyces cerevisiae*, potassium phosphate, sodium chloride and 5-caffeoylquinic acid were obtained from Sigma-Aldrich (Steinheim, Germany). The 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), magnesium chloride hexahydrate and gallic acid were purchased from Fluka Chemika (Neu-Ulm, Switzerland); sodium carbonate (anhydrous), sodium benzoate and potassium sorbate were bought from Panreac Química S.A. (Barcelona, Spain). The LIPASE-PS™ (Kit) was obtained from Trinity Biotech (Jamestown, NY). Cyanidin-3-*O*-glucoside was obtained from Polyphenols Laboratories AS (Sandnes, Norway).

Fruits

Lyophilised maqui and açaí berries were provided by Ecuadorian Rainforest LLC (Belleville, NY). Blackthorn fruit was obtained from Importaciones Samanes SL (Navarra, Spain) and lyophilised and ground later. Lemon juice was obtained from ''Verna'' lemons freshly collected from the Centre of Edafology and Applied Biology of the Segura-Spanish National Research Council (CEBAS-CSIC) experimental farm (La Matanza, Santomera, Murcia, SE Spain; 38°6′14″ N, 1°1′59″ W).

Experimental design

The new isotonic blends were tested and compared with six different and popular commercial isotonic drinks: Aquarius®, Gatorade®, Powerade®, Isostar®, Hacendado® and Ev$_2$o light®. The composition of the new isotonic beverage (per 100 mL) was: 80 mL of water, 20 mL of lemon juice [pH: 2.37, titratable acidity (TA): 5.4%], 7.5 g (w/v) of sucrose, 5 g of each lyophilised berry, 20 mg of NaCl, 6 mg of potassium phosphate and 33 mg of potassium sorbate and 16 mg of sodium benzoate as regulated preservatives (Spanish Law, Royal Decree 142/2002). One isotonic beverage was assayed without berries (lemon juice only) as a control. In addition, four isotonic drinks with control citric acid buffer, 5 g/100 mL (pH: 2.31, TA: 5.1%), replacing lemon juice, were prepared (three with berries, one without berries) using the same proportions. Samples were filtered through sterile cheesecloth (Texpol, Barcelona, Spain). The mixtures and controls were stored in transparent glass vials (56 × 18 mm Ø; volume 10 mL) with plastic screw caps. Triplicate solutions were prepared for each experiment and all analytical measurements were performed in triplicate.

The samples were labelled as follows: A (isotonic citric acid drink), AM (isotonic drink with citric acid + maqui berry), AA (isotonic drink with citric acid + açaí berry), AB (isotonic drink with citric acid + blackthorn berry), L (isotonic lemon juice drink), LM (isotonic drink with lemon juice + maqui berry), LA (isotonic drink with lemon juice + açaí berry) and LB (isotonic drink with lemon juice + açaí berry).

pH, TA and total soluble solids

The pH, TA and total soluble solids (TSS) were evaluated as quality indexes, following the method of Mena et al. (2011). The TA was expressed as gram of citric acid per 100 mL of sample and the TSS in °Brix.

Analysis of mineral elements

The analysis of P, S, Na, K, Ca, Mg, Se, Fe, Mn and Zn was carried out by ICP Emission Spectrometer (OES Thermo Scientific iCAP 6000 Series®; Thermo Electron Corp., Franklin, MA) for an aliquot of the extract diluted with $LaCl_3 + CsCl$, as reported previously (Dominguez-Perles et al., 2011).

Colour measurements

The colour measurements were carried out using the method of González-Molina et al. (2008). The data (CIE*L**, *a** and *b**) were recorded and processed using the Minolta Software Chromacontrol (S), for a PC-based colourimetric data system. Chroma (*C**) was calculated from $(a^{*2} + b^{*2})^{1/2}$.

Analysis of phenolic compounds by reverse-phase high-performance liquid chromatography with diode array detector

All samples were centrifuged for 5 min at 10 500 rpm (Hettich EBA 21 Centrifuge, Hettich Zentrifugen, Tuttlingen, Germany). The supernatant was filtered through a 0.45-μm NY Filter (Millex HV13, Millipore, Bedford, MA) before injection into the high-performance liquid chromatography (HPLC) system. The HPLC system was equipped with a Luna C_{18} Column (25 × 0.46 cm i.d., 5 μm particle size; Phenomenex, Macclesfield, UK) and a C_{18} SecurityGuard (4.0 × 3.0 mm) cartridge system (Phenomenex, Macclesfield, UK). Water:formic acid (99:5, v/v) and acetonitrile were used as mobile phases A and B, respectively, with a flow rate of 1 mL/min. The linear gradient started with 8% of solvent B, reaching 15% solvent B at 25 min, 22% at 55 min and 40% at 60 min, which was maintained up to 70 min. The injection volume was 20 μL. Chromatograms were recorded at 280, 320, 360 and 520 nm. Different phenolics were characterised by chromatographic comparison with analytical standards and according to previous reports (Gironés-Vilaplana et al., 2012a,b), and were quantified by the absorbance of their corresponding peaks. Anthocyanins were quantified as cyanidin 3-*O*-glucoside at 520 nm, and cinnamic acids as 5-*O*-caffeoylquinic acid at 320 nm.

Antioxidant capacity

All samples were centrifuged at 10 500 rpm (Hettich EBA 21 Centrifuge) for 5 min at room temperature. The free radical scavenging activity was determined using the free radical DPPH$^{\bullet}$ and ABTS^{+} methods, according to Mena et al. (2011), using 96-well micro plates (Nunc, Roskilde, Denmark) and an Infinite® M200 Microplate Reader (Tecan, Grödig, Austria). The antioxidant capacity was evaluated by measuring the variation in absorbance at 515 nm after 50 min of reaction (DPPH$^{\bullet}$) and at 414 nm after 50 min (ABTS^{+}). All reactions were started by adding 2 μL of the corresponding diluted sample to the well containing the stock solution (250 μL). The results were expressed as millimolar of Trolox.

The superoxide radical ($O_2^{\bullet -}$) scavenging activity was determined spectrophotometrically, in a 96-well plate reader, by monitoring the effect of controls and blends on the $O_2^{\bullet -}$ induced reduction of NBT at 560 nm in an NADH/PMS system, according to a described procedure (Ferreres et al., 2009). The samples were diluted one-eighth in phosphate buffer (19 mM, pH 7.4). The experiments were performed in triplicate.

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Total phenolic content determined with the Folin–Ciocalteu reagent

The total phenolic content (TPC) was determined by the Folin–Ciocalteu reagent method, adapted to a microscale according to a described procedure (Mena et al., 2012). The results were expressed as milligram of gallic acid equivalents (GAE) per 100 mL.

Lipase inhibitory effect

Lipase-PS™ reagents were obtained from Trinity Biotech (Procedure No. 805, Trinity Biotech, Jamestown, NY). Lipase activity was determined as previously described (Moreno et al., 2003), adapted to a microscale for 96-well micro plates (Nunc, Roskilde, Denmark), in an Infinite® M200 Microplate Reader (Tecan, Grödig, Austria). In each well, aliquots (3.75 µL) of lipase standard and samples (using water as blank) were added to 225 µL of reconstituted substrate solution, mixed gently and incubated for 5 min at 37 °C. Activator reagent (75 µL) was added and mixed, and the samples were incubated again for 3 min at 37 °C. The recorded rate of increase in absorbance at 550 nm – due to the formation of quinone diimine dye – was used to determine the pancreatic lipase activity in the samples prepared.

Alpha-glucosidase inhibitory activity

The alpha-glucosidase inhibitory activity assay used was a modification of a previously reported procedure (Chan et al., 2010). All samples were diluted one-half in buffer. The absorbance of 4-nitrophenol, released from 4-nitrophenyl alpha-*D*-glucopyranoside, at 400 nm was measured. The increase in absorbance was compared with that of the control (buffer instead of sample solution) to calculate the inhibitory activity.

Statistical analysis

The data shown are mean values ($n = 3$). All data were subjected to analyses of variance and a multiple range test (Tukey's test), using PASW Statistics 18 Software (formally SPSS Statistics Software; Somers, NY). Pearson's correlation analysis was performed to corroborate relationships between selected parameters.

Results and discussion

Quality parameters

The citric acid solution was created to fit the quality parameters of the lemon juice, although these parameters varied in the final isotonic beverages – between the lemon–berry blends and their respective citric acid controls. The pH values differed slightly among the samples, generally being lower in the new isotonic blends (Table 1): the isotonic citric acid control had the lowest pH (2.35) and the commercial drink 4 beverage the highest (3.83). However, all the values were within an acceptable range. The TA was significantly higher in the new blends than in the commercial samples (Table 1), probably due to the high TA values of the lemon juice (Gironés-Vilaplana et al., 2012a; González-Molina et al., 2012). Samples with blackthorn exhibited the highest values (1.08 and 1.22, respectively). Nevertheless, all the parameters were within the normal and acceptable range for these kinds of drinks (Jain et al., 2012). The TSS contents of the commercial drinks were significantly lower than those measured in the mixtures, due to the fact that in the blends with lemon more natural sugars from the powdered fruits are present, whereas in the commercial beverages there is no natural fruit in the formulation (Table 1).

Table 1. The pH, TA and TSS of the commercial isotonic drinks and newly designed beverages.

Isotonic beverage	pH	TA	TSS
Aquarius®	2.73 ± 0.04e	0.23 ± 0.01ab	6.47 ± 0.15c
Gatorade®	3.15 ± 0.01g	0.28 ± 0.01b	6.47 ± 0.06c
Powerade®	3.46 ± 0.01i	0.38 ± 0.00c	7.67 ± 0.06d
Isostar®	3.83 ± 0.01j	0.27 ± 0.01ab	7.57 ± 0.06d
Hacendado®	2.64 ± 0.00c	0.22 ± 0.00ab	5.00 ± 0.00b
Ev_2o light®	3.32 ± 0.02h	0.22 ± 0.01a	3.37 ± 0.06a
Citric acid control	2.35 ± 0.06a	0.89 ± 0.01e	7.53 ± 0.15d
Citric acid + açaí	2.45 ± 0.00b	0.88 ± 0.02e	10.47 ± 0.06f
Citric acid + blackthorn	2.62 ± 0.01c	1.08 ± 0.02g	9.13 ± 0.06e
Citric acid + maqui	2.66 ± 0.01cd	0.95 ± 0.01f	9.10 ± 0.00e
Lemon control	2.61 ± 0.00c	0.77 ± 0.02d	7.92 ± 0.03d
Lemon + açaí	2.72 ± 0.01de	0.82 ± 0.01d	10.70 ± 0.26f
Lemon + blackthorn	2.74 ± 0.01e	1.22 ± 0.00h	9.60 ± 0.02e
Lemon + maqui	2.88 ± 0.01f	0.82 ± 0.06d	9.13 ± 0.45e
LSD, $p < 0.05$	0.020	0.015	0.148

Means ($n = 3$) in the same column followed by different letters are significantly different at $p < 0.05$, according to Tukey's test. LSD, Least significant difference.

Mineral nutrients

We determined the mineral content in the new isotonic blends and commercial drinks (Table 2). Some minerals (Ca, K, Mg, Na and P) are present at relatively high levels in cells and tissues, while others are trace elements (Fe, Mn, Se and Zn) which, although present in smaller quantities, are crucial for biological processes (Speich et al., 2001). Their presence in sport drinks is important in order to avoid dehydration and electrolyte imbalance during exercise (Shirreffs, 2009).

Our most-significant findings are the relative concentrations of Na and K in the blended drinks. The Na contents of the lemon–berry drinks were quite similar to those of the respective citric acid controls. The higher K concentrations of the lemon–berry drinks (maximum of 619.30 mg/L, for lemon + blackthorn) were probably due to the K content of the fruits. Potassium levels up to 138 mg/100 g in lemon have been reported (González-Molina et al., 2010) and blackthorn is also high in K (Marakoglu et al., 2005). The lower Na:K ratios of the lemon–berry blends (mean value of 0.51 versus 0.99 for the citric acid blends) may imply positive consequences for health. Sodium and K are the main electrolytes involved in the control of extracellular and intracellular liquid volumes, respectively (Von Duvillard et al., 2004) and are important for acid–base homeostasis. Dietary patterns of western societies often show excess Na and inadequate K intake, factors causally related to cardiovascular diseases (O'Donnell et al., 2011; Rodriguez et al., 2011) and all-cause mortality (Yang et al., 2011). The new lemon–berry blends, thus, show some advantages with respect to the citric acid controls: the use of lemon as a natural ingredient (versus citric acid), a source of flavanones and vitamin C and, most importantly, a better Na:K ratio. In all cases, the commercial drinks had much-higher Na:K ratios (mean value of 5.14), due to their high content of Na.

The lemon–berry blends showed higher concentrations of Ca and P than the citric acid controls, due to the lemon contribution (González-Molina et al., 2010). The Mg concentrations were similar between groups and, within each group, blackthorn and maqui gave the highest contributions.

In the case of the commercial drinks, a greater heterogeneity in the mineral concentrations was observed, with commercial drink 4 showing peak amounts of Ca, Mg and P while the lowest concentrations corresponded to commercial drink 2 for Ca, commercial drink 5 for Mg and commercial drink 3 for P.

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Table 2. Mineral composition of commercial isotonic drinks and newly designed beverages.

Isotonic beverage	Ca	Fe	K	Mg	Mn	Na	P	Se	Zn	Na/K ratio
Aquarius®	33.22 ± 0.36b	0.08 ± 0.00a	26.44 ± 0.78a	8.15 ± 0.36a	0.06 ± 0.00a	253.60 ± 2.40d	10.74 ± 0.12ab	0.09 ± 0.00ef	0.02 ± 0.02a	9.60
Gatorade®	3.78 ± 0.27a	0.03 ± 0.01a	113.10 ± 0.14bc	47.79 ± 0.11bc	0.27 ± 0.00bc	491.05 ± 5.59g	100.55 ± 1.77h	0.08 ± 0.00e	0.09 ± 0.11a	4.34
Powerade®	24.22 ± 0.33b	0.01 ± 0.00a	118.60 ± 1.56bc	8.56 ± 0.05a	0.06 ± 0.00a	519.70 ± 4.67h	6.37 ± 0.01a	0.10 ± 0.00g	0.01 ± 0.00a	4.38
Isostar®	289.40 ± 3.39h	0.25 ± 0.00b	153.80 ± 0.99c	130.40 ± 0.71g	0.65 ± 0.00e	463.40 ± 0.71f	170.45 ± 3.61i	0.09 ± 0.00f	0.02 ± 0.00a	3.01
Hacendado®	24.57 ± 0.15b	0.03 ± 0.00a	49.58 ± 0.82a	2.87 ± 0.16a	0.02 ± 0.00a	391.95 ± 0.49e	32.73 ± 0.05d	0.06 ± 0.00d	0.01 ± 0.00a	7.91
Ev-o light®	91.00 ± 0.65def	0.02 ± 0.00a	129.30 ± 0.57bc	42.02 ± 0.06b	0.24 ± 0.00b	206.10 ± 0.85bc	17.31 ± 0.01c	0.05 ± 0.00c	0.01 ± 0.01a	1.59
Citric acid control	63.51 ± 0.69c	0.10 ± 0.11a	106.45 ± 1.48b	52.04 ± 0.62c	0.26 ± 0.00bc	207.20 ± 1.70bc	15.69 ± 0.31bc	0.03 ± 0.00a	0.84 ± 0.02bc	1.95
Citric acid + açaí	81.80 ± 3.71de	0.62 ± 0.02d	241.60 ± 9.90d	69.68 ± 2.16d	1.14 ± 0.03h	222.85 ± 7.42bc	32.08 ± 0.99d	0.05 ± 0.00c	1.72 ± 0.39d	0.92
Citric acid + blackthorn	93.07 ± 2.61ef	0.09 ± 0.00a	462.20 ± 19.23g	82.91 ± 3.54ef	0.57 ± 0.01d	215.90 ± 8.91bc	56.15 ± 0.40f	0.04 ± 0.00b	1.16 ± 0.22cd	0.47
Citric acid + maqui	96.10 ± 4.84f	0.43 ± 0.01c	351.50 ± 24.75f	79.26 ± 2.96e	0.81 ± 0.02f	212.85 ± 7.42a	46.01 ± 2.31e	0.04 ± 0.00b	1.17 ± 0.02cd	0.61
Lemon control	79.90 ± 0.54d	0.04 ± 0.01a	297.60 ± 0.99e	53.61 ± 0.35c	0.31 ± 0.00c	194.55 ± 1.63a	27.43 ± 0.08d	0.03 ± 0.00a	0.58 ± 0.31abc	0.65
Lemon + açaí	94.83 ± 1.98f	0.73 ± 0.02d	321.00 ± 16.12ef	70.25 ± 2.76d	1.16 ± 0.04h	214.20 ± 5.80bc	45.35 ± 1.51e	0.05 ± 0.00c	1.12 ± 0.01cd	0.67
Lemon + blackthorn	118.65 ± 3.61g	0.13 ± 0.00ab	619.30 ± 14.85g	84.06 ± 2.28ef	0.59 ± 0.01de	219.90 ± 6.65bc	70.09 ± 1.12g	0.04 ± 0.00b	0.35 ± 0.02ab	0.36
Lemon + maqui	120.70 ± 6.93g	0.61 ± 0.03d	594.30 ± 12.02g	89.67 ± 5.47f	0.90 ± 0.01g	204.90 ± 13.58bc	68.42 ± 0.74g	0.04 ± 0.00b	1.06 ± 0.01c	0.34
LSD, $p<0.05$	2.951	0.031	11.063	2.236	0.015	6.080	1.384	0.002	0.151	

Means ($n=3$) in the same column followed by different letters are significantly different at $p<0.05$, according to Tukey's test. Mineral concentration is expressed as mg/L. LSD, Least significant difference.

Small quantities of Fe, Mn, Se and Zn were present in all the beverages: lemon + açai showed the highest concentrations of Fe (0.73 mg/L) and Mn (1.16 mg/L) while citric acid + açai had the highest concentration of Zn (1.72 mg/L). The levels of Se were similar among the lemon–berry drinks and their citric acid controls and among the commercial drinks.

These minerals are involved in physiological processes of importance to athletes, such as: muscle and heart contraction, oxygen transport and oxidative phosphorylation, enzyme activation, bone health, antioxidant capacities and immune functions (Speich et al., 2001). Their presence in the blend drinks might be beneficial for a better sporting performance, although much work remains for the elucidation of this; above all, the addition of lemon to the isotonic drinks produces a better mineral profile.

Colour parameters

Tristimulus colorimetry is considered as the best method to evaluate visible colour and it is also often used for determining the total anthocyanin content (Khandare et al., 2011). Colour is a crucial organoleptic characteristic that triggers the first response of the consumer in contact with the product. The commercially available drinks used included artificial colourants in their formulations, such as brilliant blue or sunset yellow, to make them more attractive to consumers. The newly designed isotonic blends with lemon and natural colourants from berries (anthocyanins) had a dark-red colour – of natural appearance – attractive to consumers. The colour parameters determined displayed statistically significant differences among the beverages, especially when comparing the commercial drinks with the new berry blends (Table 3). The CIEL^* values differed significantly between the control beverages (citric acid and lemon) and the new berry blends, depending on the fruit employed in their elaboration: citric acid + açaí and citric acid + blackthorn had the most-intense luminosities, followed by the lemon + blackthorn and lemon + açaí blends (Table 3). The blends with maqui were the darkest, especially lemon + maqui. Regarding CIEa^*, the citric acid and lemon juice controls displayed similar and lower values, and the new blends higher values, for this parameter. The respective citric acid control and lemon juice blends of the açaí samples had similar values, while the blackthorn samples had a more-intense red colour (higher CIEa^* parameter) and the maqui samples were the reddest, particularly the citric acid + maqui drink (Table 3). The newly designed beverages with berries also displayed higher values for the CIEb^* parameter than did the controls, indicative of a more-yellow component in the colour spectrum, especially the lemon–berry blends of açaí and maqui (Table 3). Taking into account that the Chroma parameter is related to the CIEa^* and CIEb^* values, lower values were found for the controls and commercial beverages with respect to the newly designed berry blends, as expected. The maqui-based drinks displayed the highest Chroma values, followed by the blackthorn samples and açaí blends.

Phenolic compounds: anthocyanins and cinnamic acids

The analysis of the isotonic beverages based on lemon juice and berries revealed the presence of a wide range of phenolic compounds (Figure 1). Concerning maqui, different glycosides and di-glycosides of delphinidin (A1, A2, A5, A6) and cyanidin (A3, A4, A11) were found (Table 4), in accordance with previous studies (Gironés-Vilaplana et al., 2012a,b). The açaí and blackthorn samples contained different glycosides of cyanidin, and the blackthorn drinks had two additional peonidin glycosides, as recently described for this berry (Gironés-Vilaplana et al., 2012b). Regarding the total anthocyanins, the maqui blends had

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Table 3. Colour parameters of new isotonic blends and isotonic commercial drinks.

Isotonic beverage	L^*	a^*	b^*	Chroma
Aquarius®	90.29 ± 0.04h	0.30 ± 0.01b	2.33 ± 0.01ab	2.35 ± 0.01abc
Gatorade®	89.35 ± 0.01gh	0.40 ± 0.00b	3.59 ± 0.01b	3.62 ± 0.01bc
Powerade®	87.38 ± 0.02g	−6.40 ± 0.14a	0.07 ± 0.04a	6.40 ± 0.14d
Isostar®	89.32 ± 0.04gh	0.32 ± 0.04b	3.76 ± 0.04b	3.77 ± 0.04c
Hacendado®	91.18 ± 0.02h	0.20 ± 0.01b	2.06 ± 0.01ab	2.07 ± 0.01abc
Ev$_2$o light®	90.98 ± 0.19h	0.25 ± 0.07b	2.37 ± 0.15ab	2.38 ± 0.16abc
Citric acid control	93.40 ± 0.01i	−0.24 ± 0.01b	−0.35 ± 0.05a	0.42 ± 0.05a
Citric acid + açaí	70.74 ± 0.48f	16.67 ± 0.21c	11.12 ± 0.28c	20.04 ± 0.33e
Citric acid + blackthorn	65.83 ± 0.18e	33.49 ± 0.16e	11.35 ± 0.13c	35.36 ± 0.20h
Citric acid + maqui	41.54 ± 1.63b	61.98 ± 1.10g	15.03 ± 1.46c	63.80 ± 0.73j
Lemon control	90.61 ± 0.40h	−0.10 ± 0.08b	1.55 ± 0.21ab	1.56 ± 0.20ab
Lemon + açaí	57.18 ± 0.68c	16.52 ± 0.39c	20.42 ± 0.43d	26.27 ± 0.58f
Lemon + blackthorn	60.73 ± 0.20d	29.01 ± 0.29d	14.45 ± 0.14c	32.41 ± 0.20g
Lemon + maqui	23.97 ± 1.88a	45.10 ± 0.34f	41.18 ± 3.24e	61.09 ± 2.40i

Means ($n = 3$) in the same column followed by different letters are significantly different at $p < 0.05$, according to Tukey's test. LSD, Least significant difference. *$p < 0.05$.

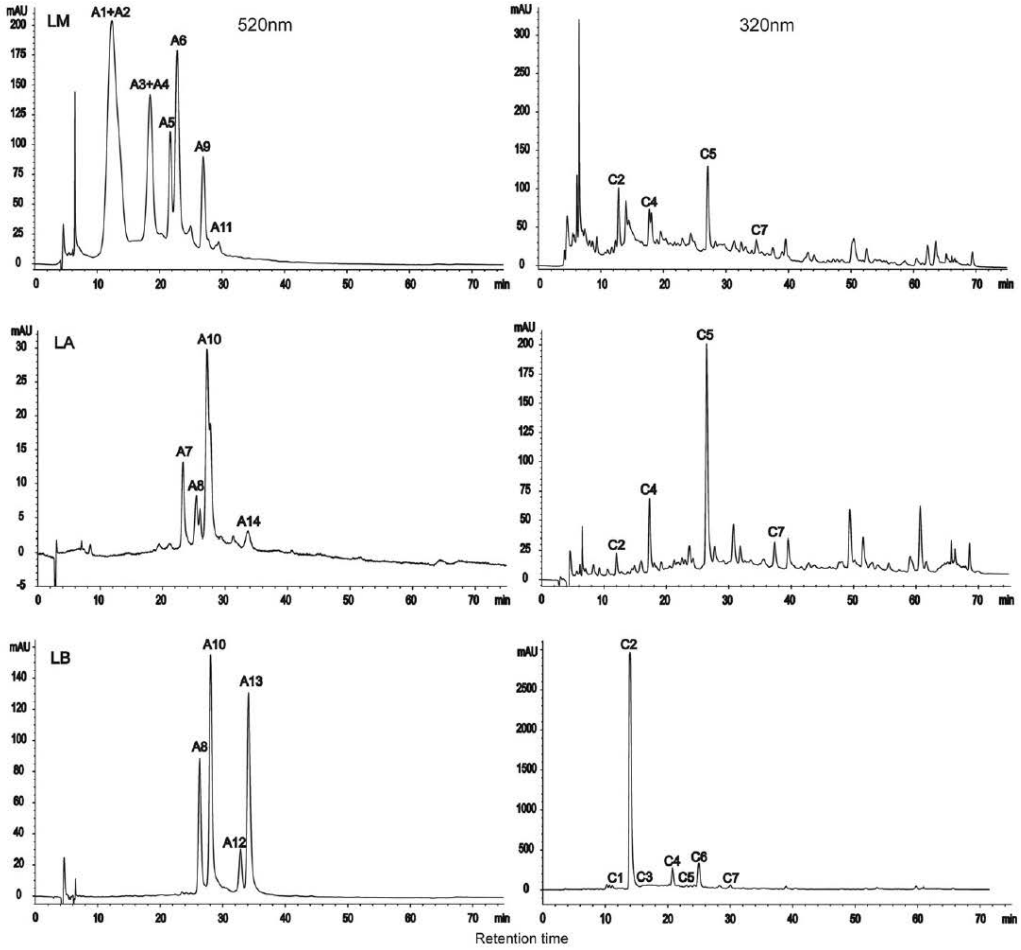

Figure 1. Chromatograms of newly designed isotonic drinks at 520 nm (anthocyanins) and 320 nm (hydroxycinnamic acid derivatives). LA (lemon–açaí isotonic blend), LB (lemon–blackthorn isotonic blend) and LM (lemon–maqui isotonic blend). The identities of the compounds associated with the peaks shown here are given in Table 4.

Table 4. Quantification (mg/100 mL of isotonic drink) of different phenolic compounds present in the new blends.

	Citric acid + açaí	Citric acid + blackthorn	Citric acid + maqui	Lemon juice control	Lemon juice + açaí	Lemon juice + blackthorn	Lemon juice + maqui
Anthocyanins							
A1 Delphinidin 3-sambubioside-5-glucoside	–	–	17.91 ± 1.78*	–	–	–	16.06 ± 0.42*
A2 Delphinidin 3,5-diglucoside	–	–	–	–	–	–	–
A3 Cyanidin 3,5-diglucoside	–	–	10.47 ± 0.34*	–	–	–	8.96 ± 0.31*
A4 Cyanidin 3-sambubioside-5-glucoside	–	–	–	–	–	–	–
A5 Delphinidin 3-sambubioside	–	–	5.69 ± 0.04	–	–	–	5.78 ± 0.57
A6 Delphinidin 3-glucoside	–	–	1.93 ± 0.02	–	–	–	1.99 ± 0.34
A7 Cyanidin 3-galactoside	0.23 ± 0.05	–	–	–	0.34 ± 0.04	–	–
A8 Cyanidin 3-glucoside	0.11 ± 0.03	1.44 ± 0.10	–	–	0.19 ± 0.06	1.08 ± 0.04	–
A9 Cyanidin 3-sambubioside	–	–	4.68 ± 0.35	–	–	–	3.55 ± 0.71
A10 Cyanidin-3-rutinoside	0.44 ± 0.05	2.95 ± 0.22	–	–	0.40 ± 0.06	2.67 ± 0.19	–
A11 Cyanidin 3-glucoside 5-rhamnoside	–	–	1.84 ± 0.02	–	–	–	1.24 ± 0.08
A12 Peonidin-3-glucoside	–	0.34 ± 0.05	–	–	–	0.34 ± 0.08	–
A13 Peonidin-3-rutinoside	–	2.91 ± 0.23	–	–	–	2.30 ± 0.44	–
Total anthocyanins	0.74 ± 0.03	7.64 ± 0.19	42.52 ± 1.57	–	0.93 ± 0.36	6.39 ± 0.69	37.58 ± 2.79
Hydroxycinnamic acid derivatives							
C1 Caffeoyldihidrocaffeoylquinic acid	–	1.35 ± 0.29	–	–	–	1.16 ± 0.02	–
C2 3-caffeoylquinic acid	0.75 ± 0.02	55.51 ± 0.34	0.35 ± 0.10	–	0.83 ± 0.02	49.99 ± 0.56	0.52 ± 0.13
C3 3-*p*-coumarylquinic acid	–	3.03 ± 0.51	–	–	–	2.04 ± 0.07	–
C4 4-caffeoylquinic acid	0.12 ± 0.02	1.31 ± 0.06	0.37 ± 0.04	–	0.17 ± 0.04	1.06 ± 0.49	0.38 ± 0.18
C5 5-caffeoylquinic acid	1.96 ± 0.13	2.84 ± 0.05	0.85 ± 0.04	0.19 ± 0.03	1.90 ± 0.06	2.67 ± 0.37	0.90 ± 0.09
C6 3-feruloylquinic acid	–	0.44 ± 0.06	–	–	–	0.57 ± 0.02	–
C7 Sinapic acid	–	–	–	0.16 ± 0.09	0.23 ± 0.10	0.25 ± 0.04	0.16 ± 0.08
Total hydroxycinnamic acid derivatives	2.83 ± 0.16	64.48 ± 1.09	1.57 ± 0.01	0.35 ± 0.03	3.13 ± 0.07	57.74 ± 0.60	1.96 ± 0.11

Values are the mean ± standard deviation ($n = 3$). *Anthocyanins A1 + A2 and A3 + A4 were quantified together.

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significantly higher concentrations than the rest of the samples, the peak formed by the co-eluting delphinidin 3-sambubioside-5-glucoside + delphinidin 3,5-diglucoside being the most abundant. The blackthorn beverages showed a noticeable quantity of anthocyanins, the majority being cyanidin 3-rutinoside and peonidin 3-rutinoside, whereas the açaí blends – in general – had meagre amounts (Table 4). To determine the relationship between the CIE*Lab** parameters and the total anthocyanins content, a Pearson's correlation was carried out. The total anthocyanins amount was correlated strongly with CIE*a** (redness) and Chroma ($r^2 = 0.883$ and $r^2 = 0.891$, $p < 0.001$, respectively), and was correlated negatively with CIE*L** ($r^2 = -0.830$, $p < 0.001$); hence, the isotonic blends with higher concentrations of anthocyanins had higher values of CIE*a** and Chroma and lower CIE*L** values (Table 3).

Concerning the hydroxycinnamic acid derivatives, greater quantities were found in the blackthorn samples, due to a large peak of 3-*O*-caffeoylquinic acid (Table 4). Likewise, these drinks also exhibited a higher number of different phenolic acids. In the açaí and maqui samples, 3-*O*-caffeoylquinic acid, 4-*O*-caffeoylquinic acid and 5-*O*-caffeoylquinic acid were detected also, but in lower amounts. In the new isotonic beverage made using only lemon juice, 5-*O*-caffeoylquinic acid and sinapic acid were found, with more compounds being detected but not quantified, such as flavanones (data not shown), since the amounts were not significant (0.51–0.93 mg/100mL).

Antioxidant capacity

Intense, strenuous exercise has been highlighted recently as a critical biochemical variable linked to oxidative stress and negative redox balance (Medina et al., 2012). In this aspect, isotonic drinks with antioxidant capacities from berries and lemon juice might help to regulate some oxidative biochemical disorders provoked by acute exercise. The antioxidant capacity of all the samples was measured by different methods [$ABTS^+$, $DPPH^\cdot$ and superoxide radical ($O_2^{\bullet -}$) scavenging assays], in order to compare the diverse reactivity of the samples in the different assays: aqueous medium ($ABTS^+$), methanolic medium ($DPPH^\cdot$) and ROS produced in human cells ($O_2^{\bullet -}$). Each antioxidant in a complex mixture (food matrix) has a different activity pattern for each method, resulting in different data for each assay. Therefore, to be sure that a sample possesses a significantly higher antioxidant capacity, several assays should be used.

With respect to $ABTS^+$, the samples containing berries showed a good antioxidant capacity (Table 5). The new isotonic blends with maqui berry displayed the highest values (8.35 ± 0.55 mM Trolox and 7.70 ± 0.26 mM Trolox for acid citric + maqui and lemon + maqui, respectively), supporting the strong *in vitro* antioxidant capacity of maqui reported previously and attributed to its polyphenolic content (Céspedes et al., 2010; Gironés-Vilaplana et al., 2012a). The samples with açaí or blackthorn berries showed similar capacities, lower than those of the maqui blends (Table 5). These high values of the berry-based samples are supported by the direct correlation between the $ABTS^+$ values and the total anthocyanin content ($r^2 = 0.883$, $p < 0.001$). The controls and commercial isotonic drinks exhibited no activity.

Concerning the $DPPH^\cdot$ method, all the samples displayed activities significantly lower than those obtained using $ABTS^+$ (Table 5). An isotonic drink is a beverage formulated to rehydrate the human body after exercise, so it is normal that these samples – being aqueous – are more reactive in aqueous ($ABTS^+$) than in methanolic assays ($DPPH^\cdot$). It is important to note that the isotonic beverages containing açaí did not exhibit any scavenging capacity with respect to the $DPPH^\cdot$ radical. Nevertheless, the samples that contained a significant quantity of anthocyanins (maqui and, to a lesser degree, blackthorn) showed certain capacity, which helped produce a direct correlation between this antioxidant capacity and the total anthocyanins content ($r^2 = 0.910$, $p < 0.001$). The maqui samples were the most active again (3.07 ± 0.09 mM Trolox for citric acid + maqui and 2.86 ± 0.21 mM Trolox for lemon + maqui) (Table 5). A high $DPPH^\cdot$ scavenging capacity of maqui was reported previosuly in fruit extract (Céspedes et al., 2008) and in new blends based on lemon juice with maqui berry (Gironés-Vilaplana et al., 2012b). With respect to blackthorn, a good antioxidant capacity against $DPPH^\cdot$ was also found in fresh fruit (Barros et al., 2010). The commercial and control beverages did not possess any anti-radical capacities.

All the samples showed high activity in the superoxide ($O_2^{\bullet -}$) scavenging assay; for this reason, all the isotonic drinks were diluted one-eighth in phosphate buffer (19 mM, pH 7.4) before determining the percentage inhibition. The berry blends showed good scavenging activity (~90%), while the controls displayed certain inhibition of $O_2^{\bullet -}$ (~35%) and the commercial isotonic beverages had less activity (Table 5). A similar result was found for freeze-dried açaí, which displayed exceptional activity against superoxide radicals (Schauss et al., 2006), and the new results are also in consonance with previous findings for maqui, blackthorn

Table 5. Antioxidant capacity and TPC of new isotonic blends and isotonic commercial drinks.

Isotonic beverage	$ABTS^+$	$DPPH^\bullet$	$O_2^{\bullet -}$	Folin (TPC)
Aquarius®	0.40 ± 0.04a	0.53 ± 0.03e	17.74 ± 9.58abc	5.70 ± 0.13a
Gatorade®	0.02 ± 0.01a	0.11 ± 0.02bcd	12.60 ± 8.46a	0.92 ± 0.06a
Powerade®	0.04 ± 0.03a	0.17 ± 0.03cd	16.97 ± 8.91ab	0.48 ± 0.15a
Isostar®	0.01 ± 0.02a	0.01 ± 0.04abc	14.40 ± 9.38a	0.62 ± 4.65a
Hacendado®	0.35 ± 0.00a	0.33 ± 0.01de	10.93 ± 0.55a	4.86 ± 0.18a
Ev_2o light®	0.16 ± 0.03a	0.14 ± 0.06bcd	11.57 ± 6.91a	3.49 ± 0.15a
Citric acid control	0.07 ± 0.01a	0.16 ± 0.06cd	35.02 ± 0.58c	0.45 ± 0.03a
Citric acid + açaí	3.88 ± 0.58b	0.12 ± 0.19ab	90.79 ± 0.61d	39.31 ± 0.58b
Citric acid + blackthorn	3.09 ± 0.44b	1.64 ± 0.09f	84.79 ± 4.94d	42.72 ± 4.90bc
Citric acid + maqui	8.35 ± 0.55c	3.07 ± 0.09g	93.96 ± 0.21d	80.97 ± 2.13e
Lemon control	0.23 ± 0.03a	0.18 ± 0.01cd	32.44 ± 4.25bc	5.90 ± 0.52a
Lemon + açaí	3.57 ± 0.74b	0.23 ± 0.04a	88.43 ± 0.56d	42.10 ± 3.29bc
Lemon + blackthorn	2.97 ± 0.23b	1.73 ± 0.09f	84.83 ± 1.45d	45.41 ± 1.45c
Lemon + maqui	7.70 ± 0.26c	2.86 ± 0.21g	90.79 ± 0.83d	73.91 ± 4.65d
LSD, $p < 0.05$	0.267	0.076	5.148	1.563

$ABTS^+$ and $DPPH^\bullet$ was expressed in millimolar Trolox, $O_2^{\bullet -}$ in percent inhibition of one-eighth buffer dilution of the drinks and Folin as milligram gallic acid/100 mL of isotonic drink. Means ($n = 3$) in the same column followed by different letters are significantly different at $p < 0.05$, according to Tukey's test. LSD, Least significant difference.

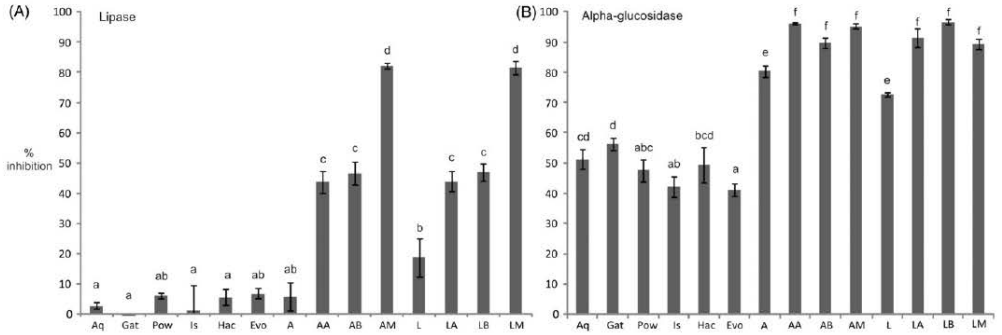

Figure 2. Inhibition of the lipase and alpha-glucosidase (diluted 0.5 times in buffer) enzyme activities (expressed as percentages). Different letters are significantly different at $p < 0.05$, according to Tukey's test. Aq (Aquarius), Gat (Gatorade), Pow (Powerade), Is (Isostar), Hac (Hacendado), Evo (Evo$_2$ light), A (isotonic citric acid drink), AA (isotonic drink with citric acid + açai berry), AB (isotonic drink with citric acid + blackthorn berry), AM (isotonic drink with citric acid + maqui berry), L (isotonic lemon juice drink), LA (isotonic drink with lemon juice + açai berry), LB (isotonic drink with lemon juice + açai berry) and LM (isotonic drink with lemon juice + maqui berry).

or açaí mixed with lemon juice (Gironés-Vilaplana et al., 2012b). In other studies on the $O_2^{\bullet-}$ scavenging activity of flavonoids, anthocyanin extracts from litchi (*Litchi chinenesis* Sonn.) also showed excellent activity ($IC_{50} = 415.8\,\mu g/mL$) (Duan et al., 2007). Nevertheless, significant correlation between the total anthocyanins and the $O_2^{\bullet-}$ scavenging activity was not found in our case. This could be due to the activity of other components in the mixtures, which we did not detect or analyse.

It is interesting to note that the commercial isotonic beverages did not display any antioxidant capacity and that the new isotonic blends behaved differently against the different reactive species, the maqui drinks being the most active in all the antioxidant assays (Table 5), likely due to their higher anthocyanin contents. Nevertheless, other phenolic compounds should be taken into consideration when this antioxidant capacity is studied.

Total phenolics determined with the Folin–Ciocalteu reagent (TPC)

The TPC results are expressed as mg of GAE per 100 mL (Table 5). The drinks which included berries in their formulation had good quantities of TPC, the maqui-isotonic blends having the greatest amounts, followed by the blackthorn-isotonic blends and the açaí-isotonic drinks (Table 5). Moreover, the new lemon + açaí and lemon + blackthorn isotonic beverages possessed the additional lemon TPC, with respect to their citric acid controls, unlike the maqui samples. As expected, the isotonic commercial beverages and controls did not display significant amounts of TPC. The TPC concentration was correlated strongly with the three antioxidant capacities assayed ($r^2 = 0.980$, 0.830 and 0.905, with $p < 0.001$; for $ABTS^+$, DPPH$^{\bullet}$ and $O_2^{\bullet-}$, respectively). However, the TPC results represent an estimation of the total phenolics because the reagent can react not only with phenolics but also with a variety of non-phenolic reducing compounds, including tertiary aliphatic amines, tertiary amine-containing biological buffers, amino acids (tryptophan), hydroxylamine, hydrazine, certain purines and other organic and inorganic reducing agents, leading to over-estimation of the phenolics content (Ikawa et al., 2003). Moreover, different phenolics can react differently to the Folin–Ciocalteu reagent, exhibiting lower absorption and hence leading to under-estimation of compounds (Ikawa et al., 2003).

Lipase inhibitory effect

The inhibition of pancreatic lipase, which splits triglycerides into absorbable glycerol and fatty acids, is the main prescribed treatment for obesity in developed countries (Moreno et al., 2003). All the new and commercial isotonic drinks were tested for their ability to inhibit pancreatic lipase *in vitro*, expressed as the percent inhibition calculated from the lowering of the relative activity compared with the activity of the control (234 U/L) (Figure 2A). The lemon juice control caused slight inhibition and the blackthorn and açaí samples displayed significant activity, the maqui blends being particularly effective (41.69 and 43.19 U/L for AM and LM, respectively). The commercial isotonic beverages and citric acid control did not show any activity (Figure 2A). The lipase activity and total anthocyanins amount were negatively and significantly correlated ($r^2 = -0.816$, $p < 0.001$); that is to say, the enzyme activity was lower in samples with higher anthocyanin contents. It has been demonstrated previously that berry polyphenols can inhibit pancreatic lipase activity *in vitro* (McDougall et al., 2009) and that phenolic extracts of maqui can reduce adipogenesis and lipid accumulation in 3T3-L1 adipocytes (Schreckinger et al., 2010). Therefore, maqui berries seem to be a potent inhibitor of pancreatic lipase *in vitro* and so their dietary ingestion could be developed as a natural alternative for obesity treatment, although further *in vivo* research would need to be conducted.

Alpha-glucosidase inhibition

Inhibition of alpha-glucosidase is considered one of the effective measures for regulating type II diabetes, by control of glucose absorption (Wang et al., 2012). The percent inhibition of this enzyme by all the isotonic drinks – diluted 1:1 with phosphate buffer (10 mM, pH 7.0) – is shown in Figure 2(B). The new isotonic blends with berries displayed significant activity (~90%), followed by the citric acid and lemon juice controls (80.3 and 72.6%, respectively). The commercial isotonic drinks only inhibited the enzyme activity by ~50%. In earlier work, berry polyphenols from blackcurrant and rowanberry were proposed as a dietary support for type 2 diabetics, to exercise glycaemic control (Boath et al., 2012). Greater inhibition of alpha-glucosidase by blueberry peel components has been found also (Wang et al., 2012). The *in vitro* anti-diabetic effects of maqui extracts have also been described (Rubilar et al., 2011), as well as

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the *in vitro* and *in vivo* effects of their anthocyanins (Rojo et al., 2011). In conclusion, the natural berries maqui, açaí and blackthorn have potential for new applications in food and industrial products (i.e. dietary supplements or coadjuvants as strong alpha-glucosidase inhibitors), particularly in isotonic beverages and other functional products.

Conclusions

New isotonic beverages were more effective than commercial isotonic drinks in terms of antioxidant capacity, TPC, minerals and biological activity, while also possessing good organoleptic properties in terms of colour. Of the studied red berries, is it noteworthy that maqui displayed the highest antioxidant capacity and the highest alpha-glucosidase and lipase inhibitory activities, due to its anthocyanins content. Among the new beverages, lemon juice is an interesting option as a natural juice which can improve the bodily Na/K balance. These new isotonic blends can be useful for equilibrating the redox balance during acute and intense exercise and for habitual sportsmen and sportswomen that need to restore their electrolytic balance after prolonged stress. Further evaluation of their biological activity and bioavailability is necessary, to verify their potential *in vivo* beneficial effects for sport, nutrition and health.

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Declaration of interest

The co-authors hereby state that there is no conflict of interest or any contractual relations or proprietary considerations that would affect the publication of the information in this article. The authors alone are responsible for the content and writing of this article.

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Evaluation of sensorial, phytochemical and biological properties of new isotonic beverages enriched with lemon and berries during shelf life

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Abstract

BACKGROUND: The aim of this work was to design new isotonic drinks with lemon juice and berries: maqui (*Aristotelia chilensis* (Molina) Stuntz), açaí (*Euterpe oleracea* Mart.) and blackthorn (*Prunus spinosa* L.), following previous research. Quality parameters, sensorial attributes, antioxidant activities ($ABTS^+$, $DPPH^\bullet$ and $O_2^{\bullet-}$ assays) and biological capacities (α-glucosidase and lipase inhibitory assays) were evaluated over 70 days of shelf-life period.

RESULTS: Maqui isotonic blends were the most active in all antioxidant assays (8.35 and 3.07 mmol L^{-1} Trolox for $ABTS^+$ and $DPPH^\bullet$), in the lipase inhibitory assay (43.19 U L^{-1}), and showed the highest total phenol content by the Folin–Ciocalteu test (80.97 mg 100 mL^{-1} gallic acid), as a result of its higher content of total anthocyanins (42.42 mg 100 mL^{-1}). Berry mixtures were also the most potent inhibitors of α-glucosidase between all samples, and displayed an attractive red colour and good sensorial attributes.

CONCLUSIONS: All the studied parameters remained quite stable during preservation, in general, and the new isotonic drinks can be useful to equilibrate redox balance in acute and intense exercise, and support weight loss programmes, avoiding triglyceride absorption and hyperglycaemia involved in obesity and diabetes mellitus, respectively. Further research *in vivo* is necessary to verify their beneficial effects for sports, nutrition and health.

Keywords: isotonic drinks; storage; berries; citrus; diabetes mellitus; obesity

INTRODUCTION

The growing interest in new added-value foods and beverages with health promoting properties has led to the development of new beverages based on different kinds of waters, juices and non-alcoholic drinks enriched with fruits, as source of nutrients and bioactive compounds. The consumer acceptance of these healthy products is subordinated to their quality and sensory characteristics that should be maintained over shelf-life. Previous research have shown that combining lemon juice [*Citrus limon* (L.) Burm. f.] with berry concentrates and powders improved the organoleptic characteristics, and the biological activity of the final product by means of antioxidant capacity and enzyme modulation,[1–5] offering new possibilities for new products to support nutrition, and public health problems associated with non-communicable diseases of adult population (i.e. obesity, diabetes mellitus, etc.).

Following our previous research on the potential benefits of lemon juice mixtures,[1–3,5] the fruits selected for designing new drinks were chosen according to their known bioactivity and composition. Lemon juice is rich in nutrients, including vitamin C, minerals, citric acid and bioactive flavonoids, which can provide health benefits beyond nutrition on cardiovascular disease, cancer, diabetes mellitus and obesity, among other chronic problems of adulthood.[6] In this case, fruits rich in anthocyanins, such as maqui, acaí and blackthorn, were studied. Maqui is a common edible berry from central and southern Chile that has also been recently reported as one of the healthiest berries, due to its bioactive components.[7,8] Various reports have linked the phenolics in maqui with its high antioxidant capacity,[9] *in vitro* inhibition of adipogenesis and inflammation,[8] protection against oxidative stress,[10] cardioprotection,[7] and *in vitro* and *in vivo* anti-diabetic effects.[9,11] Açaí is a berry from palm tree, which is a native of the Amazon River area in South America. It has recently become popular as a functional food due to its phytochemical composition, being attributed potential benefits to açaí fruits, extracts and juices: antioxidant and anti-inflammatory,[12] brain protection,[13] reduction of selected markers of the risk for metabolic disease risk,[14]

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atherosclerosis,[15] and antiproliferative properties.[16] Blackthorn is a fruit of deciduous shrubs native to Europe, mainly Spain, Portugal and Turkey.[17] It is commonly used in the preparation of jams or macerated with aniseed liqueur to obtain a digestive alcoholic drink called '*patxarán*'. Blackthorn is also cited as an astringent, diuretic and purgative,[17] and has recently been proved as an antioxidant.[18] However, a characterisation of its phenolic composition and potential for health-related benefits remain understudied.

Therefore, we aimed to design new isotonic beverages, enriched with polyphenols from lemon juice and berries, which may be useful to avoid the negative redox balance generated in intense exercise,[19] and would provide potential benefits with higher hydration capacity than juices, to support sport nutrition and healthy living. The organoleptic, phytochemical and biological characteristics of new isotonic beverages based on combinations of natural ingredients, were evaluated over a storage period of 70 days.

MATERIALS AND METHODS

Chemicals

2,2-Diphenyl-1-picrylhydrazyl radical (DPPH•) 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt ($ABTS^+$), Folin–Ciocalteu reagent, β-nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), nitrotetrazolium blue chloride (NBT), trizina hydrochloride, 4-nitrophenyl-α-D-glucopyranoside, α-glucosidase from *Saccharomyces cerevisiae*, potassium phosphate and sodium chloride were obtained from Sigma–Aldrich (Steinheim, Germany). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and magnesium chloride hexahydrate were purchased from Fluka Chemika, (Neu-Ulm, Switzerland); sodium carbonate anhydrous, sodium benzoate and potassium sorbate were bought from Panreac Química S.A. (Barcelona, Spain). LIPASE-PS™ (Kit) was obtained from Trinity Biotech (Jamestown, NY, USA). Ultrapure water was produced using a Millipore water purification system.

Fruits

Maqui and açaí lyophilised berries were provided by Ecuadorian Rainforest, LLC (Belleville, NJ, USA). Blackthorn fruit was obtained from Importaciones Samanes S.L. (Valtierra, Navarra, Spain), and lyophilised and grinded after. Lemon juice was obtained from 'Verna' lemons freshly collected from CEBAS-CSIC's experimental farm ('La Matanza', Santomera, Murcia, SE Spain; 38° 6′ 14″ N, 1° 1′ 59″ W), using a domestic squeezer ('Citromatic'; Braun Española S.A., Barcelona, Spain). Juice was stored frozen (−20°C) until used.

Experimental design

The composition of the new isotonic beverages, for 100 mL, was: 80 mL of water, 20 mL of lemon juice [pH, 2.37; titratable acidity (TA), 5.4%], 7.5 g (w/v) of sucrose, 5 g of each lyophilised berry, 20 mg of NaCl, 6 mg of potassium phosphate, and 33 mg of potassium sorbate and 16 mg of sodium benzoate as conservants (according to Spanish regulation RD 142/2002). One isotonic beverage was assayed without berries (only with lemon juice) as control. In addition, four isotonic drinks with control citric acid buffer 5 g 100 mL^{-1} (pH, 2.31; TA, 5.1%) replacing lemon juice were prepared (three with berries, one without berries), using the same proportions. Samples were filtrated later by cheesecloth from Texpol (Barcelona, Spain). Mixtures and controls were stored in transparent glass vials (56 mm × 18 mm diameter; volume 10 mL) with plastic screw caps, and stored at 18–22°C (simulation of common temperature conditions used during storage facilities in the Mediterranean area of study) in the dark for 70 days. Triplicate solutions were prepared for each experiment and all analytical measurements were done in triplicate.

Samples were labelled as follows: L (isotonic lemon juice drink), LM (isotonic lemon juice plus maqui berry drink), LA (isotonic lemon juice plus açaí berry drink), LB (isotonic lemon juice plus blackthorn berry drink), A (isotonic citric acid drink), AM (isotonic citric acid plus maqui berry drink), AA (isotonic citric acid plus açaí berry drink), and AB (isotonic citric acid plus blackthorn berry drink).

Analyses were carried out every 7 days for the first 28 days, and every 14 days during the rest of the experiment.

pH, titratable acidity, and total soluble solids

pH, titratable acidity (TA), and total soluble solids (TSS) were evaluated as quality indexes following the method reported by Mena *et al.*[20] Results were expressed as g citric acid per 100 mL of sample in TA, and°Brix in TSS.

Colour measurements

Colour measurement was determined following the method reported by González-Molina *et al.*[4] Data (CIE L^*, a^* and b^*), were recorded and processed using the Minolta Software Chromacontrol S, PC-based colorimetric data system. Hue angle (H) was calculated from $\tan^{-1}$ (b^*/a^*) and chroma (C^*) from $(a^{*2} + b^{*2})^{1/2}$.

Identification of phenolic compounds by HPLC-DAD-ESI/MSn and quantification by RP-HPLC-DAD

Chromatographic analyses for the identification were carried out on a Luna C18 column (250 × 4.6 mm, 5 mm particle size; Phenomenex, Macclesfield, UK). Water–formic acid (99:1, v/v) and acetonitrile were used as mobile phases A and B, respectively, with a flow rate of 1 mL min^{-1}. The linear gradient started with 8% of solvent B, reaching 15% solvent B at 25 min, 22% at 55, and 40% at 60 min, which was maintained up to 70 min. The injection volume was 30 µL. Chromatograms were recorded at 320 and 520 nm. The HPLC-DAD-ESI/MS*n* analyses were carried out in an Agilent HPLC 1100 series equipped with a photodiode array detector and a mass detector in series (Agilent Technologies, Waldbronn, Germany). The equipment consisted of a binary pump (model G1312A), an autosampler (model G1313A), a degasser (model G1322A) and a photodiode array detector (model G1315B). The HPLC system was controlled by ChemStation software (Agilent, version 08.03). The mass detector was an ion trap spectrometer (model G2445A) equipped with an electrospray ionisation interface and was controlled by LCMSD software (Agilent, version 4.1). The ionisation conditions were adjusted at 350°C and 4 kV for capillary temperature and voltage, respectively. The nebuliser pressure and flow rate of nitrogen were 65.0 psi and 11 L min^{-1}, respectively. The full-scan mass covered the range from m/z 100 to m/z 1200. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas, with voltage ramping cycles from 0.3 to 2 V. Mass spectrometry data were acquired in the positive ionisation mode for anthocyanins and negative ionisation mode for other flavonoids. MS*n* was carried out in the automatic mode on the more abundant fragment ion in MS(n−1).

For the quantification, all samples were also centrifuged for 5 min at 10500 rpm (model EBA 21; Hettich Zentrifugen, 3000 xg,

Tuttlingen, Germany). Supernatant was filtered through a 0.45 μm NY filter (Millex HV13; Millipore, Bedford, MA, USA) before injection into the HPLC system. The HPLC system was equipped with a Luna C_{18} column (25 cm × 0.46 cm i.d., 5 μm particle size; Phenomenex) with a C_{18} security guard (4.0 × 3.0 mm) cartridge system (Phenomenex). Water–formic acid (99:5, v/v) and acetonitrile were used as mobile phases A and B, respectively, with a flow rate of 1 mL min^{-1}. The linear gradient was the same that for identification. The injection volume was 20 μL. Chromatograms were recorded at 280, 320, 360 and 520 nm. Different phenolics were characterised by chromatographic comparison with analytical standards and according to previous reports[1,3] as well as quantified by the absorbance of their corresponding peaks. Anthocyanins were quantified as cyanidin 3-*O*-glucoside at 520 nm, and cinnamic acids as 5-*O*-caffeoylquinic acid at 320 nm.

Antioxidant capacity

All samples were centrifuged at 10 480 × *g* (model EBA 21; Hettich Zentrifugen) during 5 min at room temperature. The free radical scavenging activity were determined using the free radical DPPH• and the $ABTS^+$ method in aqueous media according to Mena *et al.*[20] The antioxidant activity was evaluated by measuring the variation in absorbance at 515 nm after 50 min of reaction (DPPH•), and at 414 nm after 50 min ($ABTS^+$). Assays were measured by using 96-well micro-plates (Nunc, Roskilde, Denmark) and Infinite® M200 micro-plate reader (Tecan, Grödig, Austria). All reactions were started by adding 2 μL of the corresponding diluted sample to the well containing the stock solution (250 μL). The final volume of the assay was 252 μL. Results were expressed as mmol L^{-1} Trolox.

Superoxide radical ($O_2^{\bullet -}$) scavenging activity was determined spectrophotometrically in a 96-well plate reader by monitoring the effect of controls and blends on the $O_2^{\bullet -}$ induced reduction of NBT at 560 nm. Superoxide radicals were generated by the NADH/PMS system according to a described procedure.[21] Samples were diluted 1/8 in phosphate buffer (19 mmol L^{-1}, pH 7.4). Experiments were performed in triplicate, expressing results as inhibition percentage of NBT reduction compared to the control.

Total phenol content by the Folin–Ciocalteu reagent

Total phenolic content (TPC) was determined by the Folin–Ciocalteu reagent method adapted to a microscale according to a described procedure.[22] Results were expressed as mg per 100 mL of gallic acid equivalents (GAE).

α-Glucosidase inhibitory activity

α-Glucosidase inhibitory activity was assessed by modification of a previously reported procedure.[23] Briefly, each well contained 100 μL of 2 mmol L^{-1} 4-nitrophenyl α-D-glucopyranoside in 10 mmol L^{-1} potassium phosphate buffer (pH 7.0) and 20 μL of the samples, diluted 1/2 in buffer. The reaction was initiated by the addition of 100 μL of the enzyme solution (56.66 mU mL^{-1}). The plates were incubated at 37°C for 10 min. The absorbance of 4-nitrophenol released from 4-nitrophenyl α-D-glucopyranoside at 400 nm was measured. The increase in absorbance was compared with that of the control (buffer instead of sample solution) to calculate the inhibitory activity.

Lipase inhibitory effect

Lipase-PS™ reagents were obtained from Trinity Biotech (Procedure No. 805; Trinity Biotech, Jamestown, NY, USA). Lipase activity was determined as previously described,[24] adapted to a microscale 96-well micro plates (Nunc) in Infinite® M200 micro plate reader (Tecan, Grödig, Austria). In each well, aliquots (3.75 μL) of lipase standard and samples (using water as blank) were added to 225 μL of reconstituted substrate solution, mixed gently and incubated for 5 min at 37°C. Activator reagent (75 μL) was added and mixed, and the samples were incubated again for 3 min at 37°C. The recorded rate of increase in absorbance at 550 nm due to the formation of quinone diimine dye was used to determine the pancreatic lipase activity in the samples prepared.

Sensorial analysis

Paired comparison tests were carried out to note organoleptical differences (colour, aroma, sourness:sweetness ratio, and overall perception) between each lemon–berry isotonic blend with its respective citric acid control. Then, sensory evaluation was performed to discriminate between the colour, aroma intensity, and sourness:sweetness ratio of selected isotonic blends. The panel was also employed to determine the overall liking (affective test) for these juices. Twenty -wo panellists, aged 25–50 years, with sensory evaluation experience, were used.

Measurements were performed in individual booths with controlled illumination and temperature. The individual products were scored for new beverages sensory parameters on a scale of 1 to 5, where 1 = extremely dark colour; extremely intense aroma; extremely high sourness:sweetness ratio (higher sourness than sweetness); extremely pleasant; 2 = intense; 3 = moderate (regular colour, aroma, sourness:sweetness ratio, astringency and overall); 4 = slight; 5 = extremely light colour; extremely slight aroma; extremely low sourness:sweetness (higher sweetness than sourness); extremely unpleasant. Panellists relied on their training experience to score juices, which were presented in 50 mL plastic cups. The entire experiment was repeated three times and the sensory scores were presented as overall means.

Statistical analysis

Data shown are mean values ($n = 3$). All data were subjected to analyses of variance (ANOVA) and a multiple range test (Tukey's test), using PASW Statistics 18 software (Somers, New York, USA). Data from paired comparison test was analysed by the chi-squared test. Pearson correlation analysis was performed to corroborate relationships between selected parameters.

RESULTS AND DISCUSSION

Quality parameters

Citric acid solutions were used to simulate the conditions of the lemon juice as a food matrix. However, the physical quality parameters varied in final isotonic beverages between lemon–berry blends and their respective citric acid controls (Table 1).

Concerning both pH and TA values, relatively small differences were observed in the mixtures or the control juices over the 70 days of storage. The pH values ranged between 2.35 and 2.88, with lower pH in the citric acid samples than in beverages with lemon juice, in general, being the citric acid controls (A) the lowest value, and the maqui–lemon isotonic blend (LM) the highest. Concerning TA, the blackthorn samples (AB and LB) obtained slightly higher values than the rest (Table 1). However, all the parameters were within a normal and acceptable range for these beverages.[25]

Table 1. pH, titratable acidity, and total soluble solids of new isotonic blends during 70 days of storage

	pH		Titratable acidity (g citric acid 100 mL^{-1} juice)		Total soluble solids (°Brix, 25°C)	
Drink	Day 0	Day 70	Day 0	Day 70	Day 0	Day 70
A	2.35 ± 0.06^{a}	2.46 ± 0.02^{a}	0.89 ± 0.01^{bc}	0.87 ± 0.00^{b}	7.53 ± 0.15^{a}	8.10 ± 0.28^{a}
AA	2.45 ± 0.00^{b}	2.60 ± 0.01^{b}	0.88 ± 0.02^{bc}	0.92 ± 0.01^{b}	10.47 ± 0.06^{c}	11.35 ± 0.07^{ef}
AB	2.62 ± 0.01^{c}	2.75 ± 0.01^{c}	1.08 ± 0.02^{d}	1.10 ± 0.00^{c}	9.13 ± 0.06^{b}	10.05 ± 0.07^{cd}
AM	2.66 ± 0.01^{cd}	2.80 ± 0.01^{cd}	0.95 ± 0.01^{c}	0.92 ± 0.02^{b}	9.10 ± 0.00^{b}	9.50 ± 0.42^{bc}
L	2.61 ± 0.00^{c}	2.75 ± 0.01^{c}	0.77 ± 0.02^{a}	0.78 ± 0.01^{a}	7.92 ± 0.03^{a}	8.85 ± 0.07^{ab}
LA	2.72 ± 0.01^{d}	2.82 ± 0.01^{d}	0.82 ± 0.01^{ab}	0.91 ± 0.02^{b}	10.70 ± 0.26^{c}	12.30 ± 0.57^{f}
LB	2.74 ± 0.01^{d}	2.85 ± 0.01^{d}	1.22 ± 0.00^{e}	1.27 ± 0.01^{d}	9.60 ± 0.35^{b}	10.80 ± 0.14^{de}
LM	2.88 ± 0.01^{e}	2.97 ± 0.02^{e}	0.82 ± 0.06^{ab}	0.92 ± 0.04^{b}	9.13 ± 0.45^{b}	10.85 ± 0.07^{de}
LSD, $P < 0.05$	0.023	0.014	0.024	0.016	0.188	0.278

Values are given as the mean ± SD.
Different letters means significantly different at $P < 0.05$ according to the Tukey HSD multiple range test.
A, isotonic citric acid drink; AM, isotonic drink with citric acid plus maqui berry; AA, isotonic drink with citric acid plus açaí berry; AB, isotonic drink with citric acid plus blackthorn berry; L, isotonic lemon juice drink; LM, isotonic drink with lemon juice plus maqui berry; LA, isotonic drink with lemon juice plus açaí berry; and LB, isotonic drink with lemon juice plus blackthorn berry.

Regarding to TSS contents, an increase over storage was observed in all samples (Table 1), being part of a natural phenomenon of shelf-life attributable to the hydrolytic changes in the berry carbohydrates, (i.e. the breakdown of starch into sugars).[26]

Phenolic compounds: anthocyanins and hydroxycinnamic acids

The analysis of new isotonic drinks revealed the presence of a wide range of flavonoid phenolics (Table 2, Fig. 1). Concerning maqui blends (LM), different anthocyanins, mainly glycosides and di-glycosides of delphinidin and cyanidin were found, as expected.[1,3,27] Açaí (LA) and blackthorn (LB) samples displayed also glycosides of cyanidin, and blackthorn drinks two peonidin glycosides, as also recently reported.[3] Regarding the total anthocyanins content, the maqui blends exhibited significant higher quantity than the rest of the samples (42.42 mg 100 mL^{-1} for LM), being the co-elution peak formed by delphinidin 3-sambubioside-5-glucoside + delphinidin 3,5-diglucoside (A1+A2) the major peak (Table 2, Fig. 1). Beverages with blackthorn showed a noticeable quantity of anthocyanins (6.39 mg 100 mL^{-1} for LB), due mainly to a cyanidin 3-rutinoside and peonidin 3-rutinoside, but the açaí blends obtained a poor quantity (0.93 mg 100 mL^{-1} for LA). The total content of anthocyanins tended to decrease over the storage period of 70 days in all samples tested (Fig. 2), these decreases being more pronounced in lemon-based drinks with respect to the citric acid controls. It has been recently reported that ascorbic acid can led to the breakdown of anthocyanins,[1,4] even though only 20% (v/v) of lemon juice was used in new blends formulation, and vitamin C was not detected in significant amounts (data not shown). For this reason, the decrease of total anthocyanins in lemon isotonic drinks was only slightly higher than in the respective controls of citric acid, and of lesser extent than in previous models of lemon–berries beverages.[4,5] The LA blends displayed the most pronounced fall (by 74%), followed by LM (by 61%), AM (by 57%), LB (by 55%), AB (by 49%) and AA (by 43%), over the storage of 70 days.

Concerning hydroxycinnamic acid derivatives, it is important to note the exceptionally higher quantity in the blackthorn samples (AB and LB), due to a large peak (C2) of 3-*O*-caffeoylquinic acid (Table 2, Fig. 1),[3] and another six compounds (totalling C1–C7, 64.5 and 57.7 mg 100 mL^{-1} of hydroxycinnamic acid derivatives for AB and LB, respectively) (Table 2, Fig. 1). The 3-*O*-caffeoylquinic acid (C2), 4-*O*-caffeoylquinic acid (C4), and 5-*O*-caffeoylquinic acid (C5) were also detected and quantified in açaí and maqui beverages, but in lower amounts (2.8, 3.1, 1.6 and 1.9 mg 100 mL^{-1} of total hydroxycinnamic derivatives for AA, LA, AM and LM, respectively). Lemon juice provided 5-*O*-caffeoylquinic acid and sinapic acid to the new mixtures, with additional small amounts of compounds not quantified (only 20% of lemon juice was added in blends). In contrast to the observations in anthocyanins, only small differences were detected in total hydroxycinnamic derivatives (Fig. 2) which remained practically unchanged over the 70 days of storage in blackthorn isotonic blends, appearing little changes in the rest of the drinks.

Colour parameters

The newly designed isotonic beverages with natural anthocyanins of berries can give an attractive red/dark colour for natural appearance and consumer acceptance, for this reason, colour parameters were determined and studied over 70 days of shelf-life (Table 3). Controls without berries (A and L) displayed unacceptable colour parameters with insignificant changes during storage. The rest of samples showed appreciable increases of CIE L^* values, in general, being maqui blends the darkest, especially new blends of lemon–maqui, with negative correlation between CIE L^* and total anthocyanin content for AA ($r = -0.87$, $P < 0.01$), AB ($r = -0.85$, $P < 0.01$), AM ($r = -0.98$, $P < 0.001$), LB ($r = -0.96$, $P < 0.001$), and LM ($r = -0.92$, $P < 0.001$), suggesting that the anthocyanins degradation was related with the increased lightness (or decrease of darkness) over the shelf-life, unlike that found in other works.[28,29] Regarding CIE a^* values, açaí samples reported similar results between their respective citric acid and lemon juice blends, and the blackthorn samples were more reddish in colour (higher CIE a^*), being the maqui samples the reddest, specially the citric acid–maqui drinks (Table 3). This parameter decreased in the 70 days studied, and was strongly correlated to the total anthocyanins content in AA ($r = 0.89$, $P < 0.01$), AB ($r = 0.79$, $P < 0.05$), AM ($r = 0.90$, $P < 0.01$), LA ($r = 0.93$, $P < 0.01$), and LB ($r = 0.96$, $P <$

Table 2. Phenolic compounds identified and quantified in newly designed isotonic beverages

Phenolic compound		Tr	$[M-H]^+$	MSn	Isotonic drink LA	LB	LM
Anthocyanins							
A1	Delphinidin 3-*O*-sambubioside-5-*O*-glucoside	11.2	759	465, 597, 303	—	—	+
A2	Delphinidin 3,5-*O*-diglucoside	12.3	627	465, 303	—	—	+
A3	Cyanidin 3,5-*O*-diglucoside	19.3	611	449, 287	—	—	+
A4	Cyanidin 3-*O*-sambubioside-5-*O*-glucoside	19.7	743	449, 581, 287	—	—	+
A5	Delphinidin 3-*O*-sambubioside	21.0	597	303	—	—	+
A6	Delphinidin 3-*O*-glucoside	22.5	465	303	—	—	+
A7	Cyanidin 3-*O*-galactoside	22.9	449	287	+	—	—
A8	Cyanidin 3-*O*-glucoside	24.0	449	287	+	+	—
A9	Cyanidin 3-*O*-sambubioside	27.8	581	287	—	—	+
A10	Cyanidin-3-*O*-rutinoside	28.1	595	287	+	+	—
A11	Cyanidin 3-*O*-glucoside-5-*O*-rhamnoside	30.1	595	449, 287	—	—	+
A12	Peonidin 3-*O*-glucoside	32.9	463	301	—	+	—
A13	Peonidin 3-*O*-rutinoside	34.1	609	301	—	+	—
A14	Malvidin 3-*O*-glucoside	34.7	493	331	+	—	—
		Tr	$[M-H]^-$	MSn	Isotonic drink LA	LB	LM
Hydroxycinnamic acid derivatives							
C1	Caffeoyldihydrocaffeoylquinic acid	10.2	517	335	—	+	—
C2	3-*O*-Caffeoylquinic acid	13.4	353	191, 179	+	+	+
C3	3-*O*-*p*-Coumaroylquinic acid	15.9	337	163	—	+	—
C4	4-*O*-Caffeoylquinic acid	16.8	353	173	+	+	+
C5	5-*O*-Caffeoylquinic acid	24.7	353	191	+	+	+
C6	3-*O*-Feruloylquinic acid	26.1	367	193	—	+	—
C7	Sinapic acid	33.9	337	191	+	+	+

A1 to A14, and C1 to C7 refer to the peaks obtained during chromatography, see Fig. 1.
LA, lemon–açaí isotonic blend; LB, lemon–blackthorn isotonic blend; LM, lemon–maqui isotonic blend.

0.001), which was indicative of the influence of anthocyanins in the redness of the product. The CIE b^* value of beverages with berries was higher than in controls, but remained slightly more stable during storage, which is understandable since this parameter is associated to yellowness. Bearing into consideration that chroma is related to CIE a^* and CIE b^*, lower values found in controls with respect to the new berry-blends, was expected. The maqui samples were the most coloured (highest chroma), followed by the blackthorn drinks and the açaí beverages, and a similar decrease in CIE a^* parameter was observed over shelf-life, correlated to the total anthocyanin content: AA ($r = 0.90$, $P < 0.01$), AB ($r = 0.80$, $P < 0.05$), AM ($r = 0.92$, $P < 0.001$) and LB ($r = 0.94$, $P < 0.001$).

Is important to emphasise that, regardless of the losses in anthocyanin contents over time, the red coloration of the isotonic blends with berries remained quite in the 70 days of study, as a result of the likely formation of other coloured polymers,[30] or co-pigmentation between anthocyanins and other flavonoids that appreciably augmented visual colour and could mask the detrimental changes that took place during storage in the anthocyanin containing products.[31]

Antioxidant activity and total phenolics content

Acute and strenuous exercise has been recently highlighted as critical biochemical variables linked to oxidative stress and negative redox balance,[19] so the new isotonic blends with antioxidant capacities from berries and lemon juice, may be useful to regulate some oxidative biochemical disorders during intense exercise. With respect to $ABTS^+$, samples that contain berries showed a good antioxidant capacity, while the controls exhibited non-significant activity (Fig. 3A). New isotonic blends with maqui berry displayed the highest values (8.35 ± 0.55 mmol L^{-1} Trolox and 7.70 ± 0.26 mmol L^{-1} Trolox for AM and LM, respectively), supporting the strong *in vitro* antioxidant capacity of maqui previously reported for this method, and attributed to its polyphenolic content.[1,3] All samples remained quite stable until day 42 of preservation, following a similar decrease pattern from that day until the end of the study (Fig. 3A).

Regarding to superoxide ($O_2^{\bullet -}$) scavenging assay (Fig. 3B), all beverages reported high activity, and for this reason, were diluted 1/8 times in phosphate buffer (19 mmol L^{-1}, pH 7.4) before the calculation of the percentage of inhibition. Controls displayed significant lower activity than berry samples, keeping this anti-radical effect in all drinks very stable during the 70 days of storage. An exceptional activity against this radical was found for freeze-dried açaí.[12] Positive results were also recently found in pure lemon juice supplemented with maqui, blackthorn and açaí,[3] as well as with anthocyanin-rich extracts from litchi (*Litchi chinensis* Sonn.) or rambutan (*Nephelium lappaceum* L.) against superoxide anion.[32,33]

Low antioxidant activity was obtained in $DPPH^\bullet$ assays, as expected, since samples were aqueous and $DPPH^\bullet$ is normally

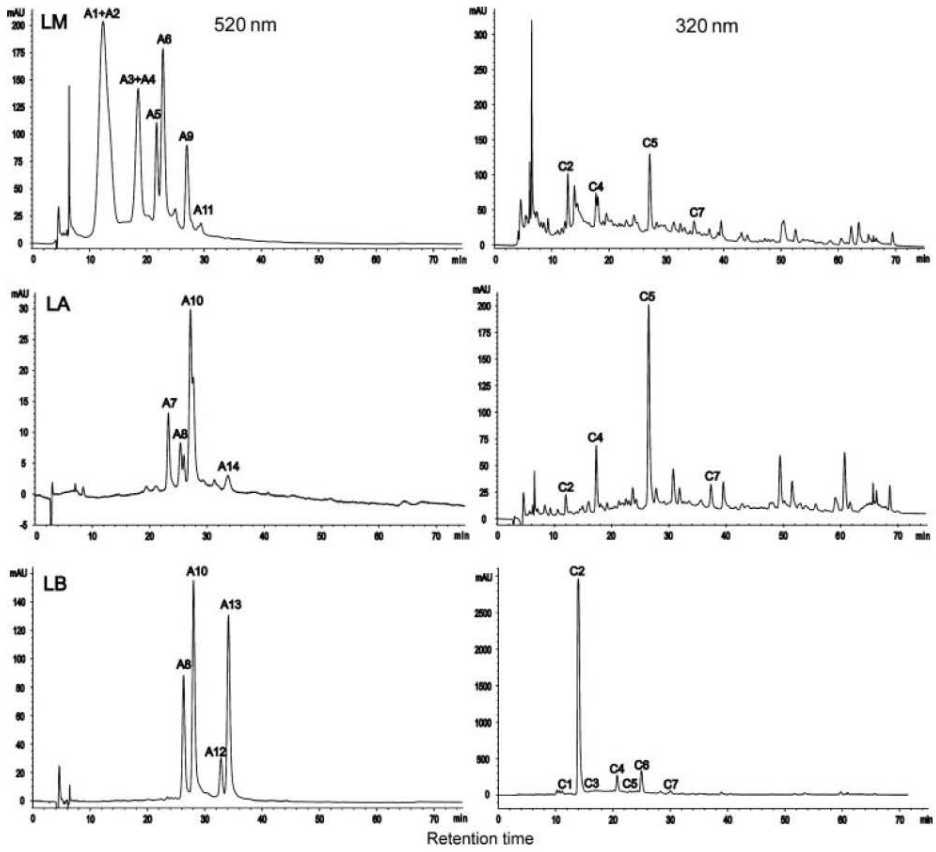

Figure 1. Chromatograms of newly designed isotonic drinks at 520 nm (anthocyanins) and 320 nm (hydroxycinnamic acid derivatives). The identities of the compounds associated with the peaks shown here are given in Table 2.

used with methanolic extracts. However, all the samples showed more stable results than in the $ABTS^+$ assay over shelf-life (Fig. 3C). The açaí beverages (AA and LA) did not display any activity, being maqui samples the most active (3.07 ± 0.09 mmol L^{-1} Trolox for AM, and 2.86 ± 0.21 mmol L^{-1} Trolox for LM), also agreeing with previous reports of maqui extracts[7] and maqui–lemon blends.[3] A good antioxidant activity was also found in blackthorn juices and fruits.[17,34]

The total phenolics content (TPC) ranged between 0.45 mg 100 mL^{-1} gallic acid in A control and 80.97 mg 100 mL^{-1} gallic acid in AM (Fig. 3D). New isotonic beverages of lemon–açaí and lemon–blackthorn showed the addition effect on TPC results of the lemon juice with respect to their citric acid controls, unlike to what happened with maqui samples. With respect to the evolution of blends over storage, all the samples showed an increase in TPC associated with the formation of secondary metabolites able to react with Folin–Ciocalteu reagent.[1] Therefore, phenolic contents determined by Folin–Ciocalteu reagent assay displayed substantially higher concentrations than those determined by HPLC methods due to a overestimation of the real phenolic content, because the reagent react not only with phenolics but also with different non-phenolic reducing compounds including tertiary aliphatic amines, tertiary amine-containing biological buffers, amino acids (tryptophan), hydroxylamine, hydrazine, certain purines, and other organic and inorganic reducing agents.[35] Furthermore, different phenolics can present different reactions with the Folin–Ciocalteu reagent, presenting lower absorption which it also leads to an underestimation of various compounds.[36]

Considering all these results, different assays can give a broader view to justify the use of multiple antioxidant tests, comparing the diverse reactivity of samples with each different assay: aqueous ($ABTS^+$), methanolic ($DPPH^\bullet$), and produced in human cells ($O_2^{\bullet-}$). It is interesting that although samples reacted differently against several reactive species, maqui samples were the most active in all antioxidant methods, also richer in total phenolic content by Folin–Ciocalteu reagent (80.97 ± 2.13 and 73.91 ± 4.65 mg 100 mL^{-1} for AM and LM, respectively). Açaí and blackthorn isotonic beverages also showed acceptable activity in $ABTS^+$ and $O_2^{\bullet-}$ assays, but we found non-significant correlations between

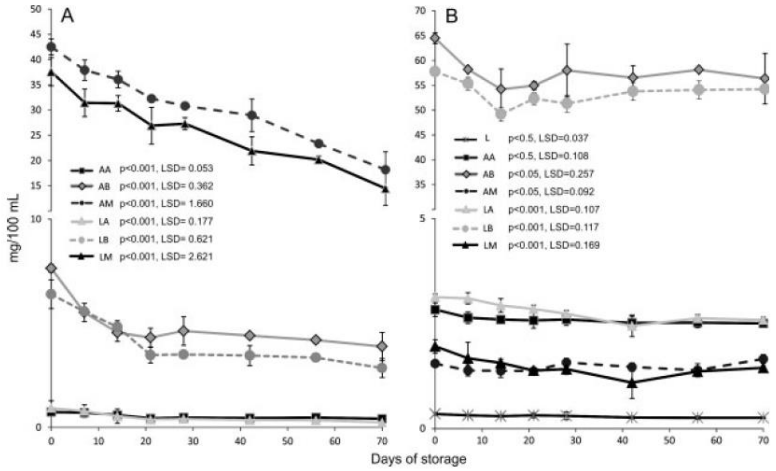

Figure 2. Total anthocyanins (A) and hydroxycinnamic acid derivatives (B) content (mg 100 mL^{-1}) of new isotonic blends during 70 days of storage. Statistical treatment notes: All data were subjected to analyses of variance (ANOVA) and a multiple range test (Tukey's test) with a significance of $P < 0.05$.

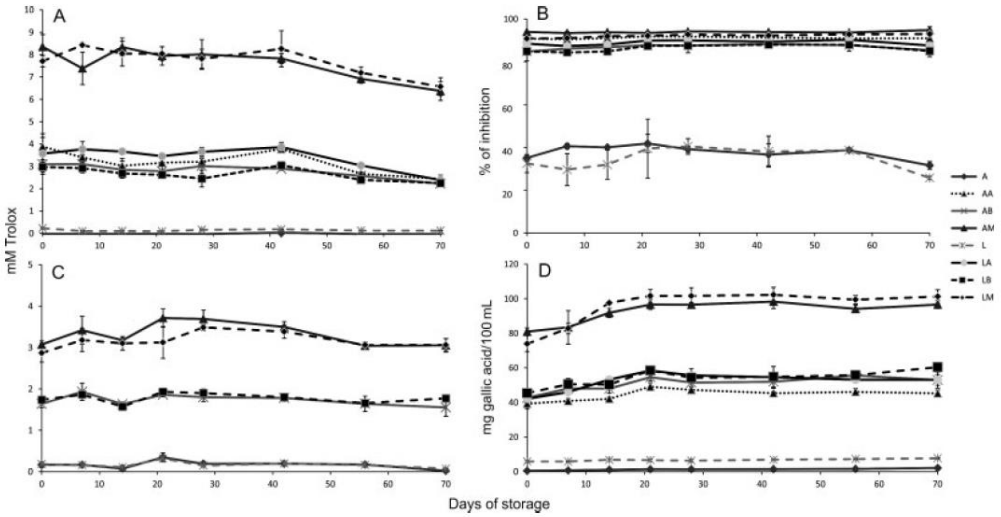

Figure 3. Antioxidant activity and total phenolic content (TPC) of new isotonic blends during 70 days of storage: (A) ABTS in mmol L^{-1} Trolox, (B) inhibition (%) of superoxide radical, (C) DPPH in mmol L^{-1} Trolox, (D) TPC by Folin–Ciocalteu in mg gallic acid 100 mL^{-1}.

total anthocyanins or hydroxycinnamic acids derivatives and the different antioxidant assays over shelf-life. This does not mean that the components or any other undetected bioactives in the matrix could have certain activity, and suggest that the phenolic compounds are not the only responsible of the antioxidant potential, but they are involved in the quality composition and in the biological activity.

α-Glucosidase inhibitory effect

The main purpose in diabetes mellitus treatment is to achieve blood glucose levels as close to normal as possible. The α-glucosidase is a key enzyme that catalyses the final step in the digestive process of carbohydrates, so the inhibition of this enzyme is one of the effective measures for regulating type 2 diabetes by controlling glucose absorption.[9] All the tested samples (Fig. 4) led to a high activity in this assay, for this reason all the isotonic drinks were diluted 0.5 times (buffer phosphate 10 mmol L^{-1}, pH 7.0). Controls of citric acid (A) and lemon juice (L) displayed 80.3% and 72.6% of enzyme inhibition, respectively, and the new isotonic drinks with berries even higher activity ranging (between 89.3% and 96.6%). This anti-diabetic effect was preserved practically during the 70 days of storage

Table 3. Stability of CIE L^*, a^* and b^* values of newly designed isotonic beverages during their shelf life

Drink and CIE parameter	Day 0	7	14	21	28	42	56	70	LSD
A									
L^*	93.41^{b}	91.63^{a}	92.92^{b}	92.65^{b}	92.68^{b}	93.04^{b}	92.59^{b}	92.89^{b}	0.239^{***}
a^*	-0.24^{a}	0.19^{f}	-0.07^{bc}	-0.04^{b}	-0.02^{cd}	0.07^{e}	0.03^{de}	0.18^{f}	0.019^{***}
b^*	-0.35^{a}	1.07^{e}	0.15^{b}	0.29^{a}	0.29^{bc}	0.46^{cd}	0.36^{c}	0.62^{d}	0.057^{***}
Chroma	0.43^{b}	1.09^{d}	0.16^{a}	0.56^{ab}	0.29^{ab}	0.47^{bc}	0.36^{b}	0.64^{c}	0.057^{***}
AA									
L^*	70.74^{a}	71.32^{ab}	73.89^{bc}	74.55^{c}	74.64^{c}	73.53^{bc}	75.26^{cd}	77.69^{d}	0.793^{***}
a^*	16.67^{g}	15.40^{f}	14.13^{e}	13.92^{e}	13.09^{d}	12.27^{c}	11.70^{b}	11.09^{a}	0.129^{***}
b^*	11.13^{ab}	11.82^{abc}	10.66^{a}	11.28^{ab}	11.99^{abc}	12.05^{bc}	12.78^{bc}	11.97^{abc}	0.385^{***}
Chroma	20.04^{c}	19.42^{c}	17.70^{b}	17.91^{b}	17.75^{b}	17.21^{ab}	17.33^{ab}	16.33^{a}	0.346^{***}
AB									
L^*	65.83^{a}	67.50^{a}	69.91^{b}	70.93^{bc}	71.97^{c}	71.26^{bc}	74.30^{d}	75.78^{d}	0.495^{***}
a^*	33.49^{d}	31.67^{d}	30.48^{cd}	30.66^{cd}	27.98^{bc}	27.99^{bc}	27.01^{ab}	24.46^{a}	0.907^{***}
b^*	11.35^{e}	10.58^{d}	9.56^{c}	9.15^{c}	8.18^{b}	8.00^{b}	7.04^{a}	6.73^{a}	0.145^{***}
Chroma	35.36^{e}	33.39^{de}	31.94^{cd}	31.99^{cd}	29.15^{bc}	29.11^{bc}	27.91^{ab}	25.37^{a}	0.893^{***}
AM									
L^*	41.54^{a}	45.91^{b}	48.14^{c}	49.05^{c}	49.51^{c}	51.77^{d}	54.17^{e}	55.57^{e}	0.623^{***}
a^*	61.98^{d}	63.87^{e}	62.77^{de}	62.66^{de}	61.42^{d}	58.36^{c}	53.85^{b}	50.45^{a}	0.558^{***}
b^*	15.03^{b}	12.79^{a}	11.57^{a}	11.64^{a}	12.27^{a}	12.73^{a}	13.63^{ab}	14.86^{b}	0.644^{***}
Chroma	63.80^{de}	65.14^{e}	63.83^{de}	63.77^{de}	62.63^{d}	59.73^{c}	55.55^{b}	52.59^{a}	0.530^{***}
L									
L^*	90.61^{a}	90.84^{ab}	91.03^{ab}	90.59^{a}	91.20^{ab}	91.42^{b}	91.32^{b}	91.39^{b}	0.191^{***}
a^*	-0.10^{a}	0.16^{b}	0.06^{b}	0.31^{c}	0.06^{b}	0.10^{b}	0.15^{b}	0.31^{c}	0.034^{***}
b^*	1.55^{a}	2.05^{bc}	1.80^{ab}	1.80^{ab}	1.78^{ab}	1.84^{b}	1.99^{bc}	2.20^{c}	0.079^{***}
Chroma	1.56^{a}	2.05^{bc}	1.80^{ab}	1.83^{ab}	1.78^{ab}	1.84^{b}	1.99^{bc}	2.23^{c}	0.078^{***}
LA									
L^*	57.18^{ab}	59.39^{cd}	58.73^{bc}	58.94^{bcd}	56.28^{a}	59.94^{cd}	61.03^{d}	58.62^{bc}	0.679^{***}
a^*	16.52^{d}	14.82^{c}	14.01^{b}	13.90^{b}	13.66^{b}	12.35^{a}	11.90^{a}	11.89^{a}	0.242^{***}
b^*	20.42^{a}	21.08^{ab}	21.43^{ab}	22.20^{b}	24.17^{c}	22.13^{b}	22.45^{b}	24.46^{c}	0.404^{***}
Chroma	26.27^{abc}	25.76^{ab}	25.60^{a}	26.20^{abc}	27.76^{c}	25.34^{a}	25.41^{a}	27.20^{bc}	0.492^{***}
LB									
L^*	60.73^{a}	63.54^{b}	68.07^{c}	70.41^{cd}	72.47^{d}	75.14^{e}	75.83^{e}	77.29^{e}	0.773^{***}
a^*	29.01^{e}	27.04^{de}	24.73^{cde}	23.50^{bcd}	22.27^{abc}	19.55^{ab}	18.36^{a}	19.22^{ab}	1.305^{***}
b^*	14.45^{c}	13.48^{bc}	11.42^{abc}	9.95^{abc}	8.99^{ab}	7.95^{a}	7.90^{a}	7.05^{a}	1.490^{***}
Chroma	32.41^{d}	30.21^{cd}	27.24^{bcd}	25.53^{abc}	24.03^{ab}	21.11^{a}	20.22^{a}	20.49^{a}	1.608^{***}
LM									
L^*	23.97^{a}	27.92^{ab}	29.80^{ab}	31.60^{ab}	29.22^{ab}	33.40^{b}	33.10^{b}	33.78^{b}	2.543^{***}
a^*	45.10^{ab}	46.37^{abc}	48.08^{bc}	48.57^{bc}	46.20^{abc}	46.52^{abc}	44.75^{a}	44.95^{ab}	0.944^{***}
b^*	41.18^{a}	42.41^{a}	40.43^{a}	38.72^{a}	45.07^{a}	34.59^{a}	38.25^{a}	39.02^{a}	6.390^{***}
Chroma	61.09^{a}	62.89^{a}	62.95^{a}	61.26^{a}	64.71^{a}	57.94^{a}	59.28^{a}	59.93^{a}	3.880^{***}

A, isotonic citric acid drink; AM, isotonic drink with citric acid plus maqui berry; AA, isotonic drink with citric acid plus açaí berry; AB, isotonic drink with citric acid plus blackthorn berry; L, isotonic lemon juice drink; LM, isotonic drink with lemon juice plus maqui berry; LA, isotonic drink with lemon juice plus açaí berry; and LB, isotonic drink with lemon juice plus açaí berry.
Different lowercase letters means significant differences at $p < 0.05$ according to Tukey HSD Multiple Range Test. Significance level (ANOVA) at $p < 0.001$ (***).

(Fig. 4). In other studies, berry polyphenols from rowanberry and blackcurrant has been reported as dietary products for type 2 diabetics to exercise glycaemic control.[37] The anti-diabetic effects of maqui have been also described *in vitro* in extracts,[9] and *in vitro* and *in vivo* for its anthocyanins.[11] In summary, berry polyphenols from maqui, açaí and blackthorn used in functional food developments, such as isotonic beverages, may offer dietary and convenient alternatives to control hyperglycaemia in diabetic patients and in subjects who wants to lose weight with the help of exercise.

Lipase inhibition

Pancreatic lipase, the principal lipolytic enzyme synthesised and secreted by the pancreas, plays a key role in the efficient digestion of triglycerides. For that, the inhibition of this enzyme is the main treatment of obesity in developed countries.[24] All new isotonic beverages were tested for their ability to inhibit pancreatic lipase *in vitro* (Fig. 5), expressed in % of inhibition calculated from the lowering of relative activity compared with the activity of the standard from Lipase-PS™ kit (234 U L^{-1}) [at the beginning (day 0) and at the end (day 70)], to find significant differences:

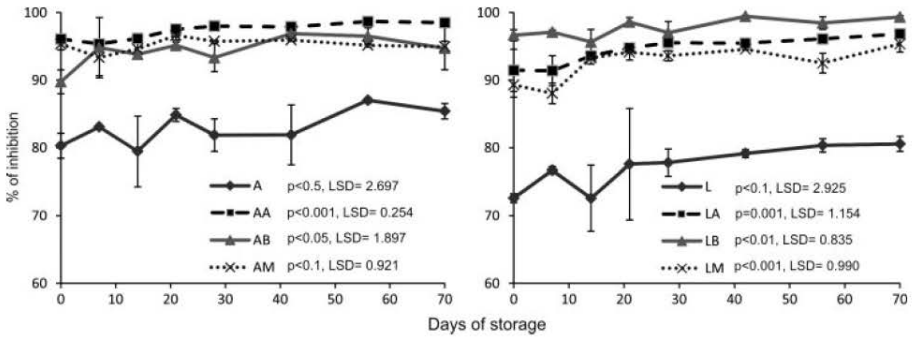

Figure 4. Inhibition (%) of α-glucosidase of all samples diluted 0.5 times in buffer during 70 days of storage. Statistical treatment notes: All data were subjected to analyses of variance (ANOVA) and a multiple range test (Tukey's test) with a significance of $P < 0.05$.

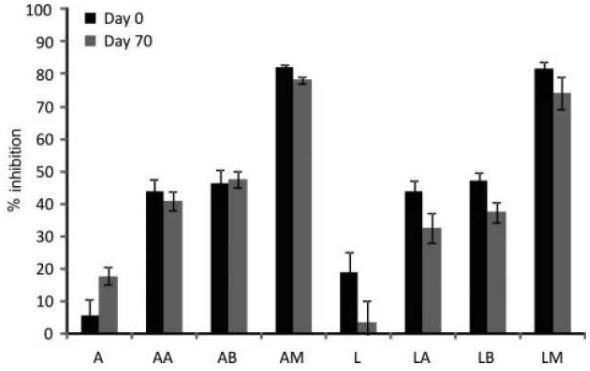

Figure 5. Inhibition (%) of lipase of all samples at the beginning (day 0) and at the end (day 70) of storage.

controls of citric acid (A) did not shown any activity at day 0, but displayed a little increase in day 70; lemon juice controls (L) caused a slight inhibition at day 0, but this inhibition was lower at day 70; blackthorn and açaí samples displayed significant activity, showing a small decrease in day 70; and maqui blends were particularly effective (only 41.69 and 43.19 U L^{-1} for AM and LM, respectively) and remained quite stable during storage. Although lemon control was more effective than citric acid control, lemon–berries beverages displayed stronger inhibitions of the enzyme than their respective citric acid–berries blends over shelf-life. It has been previosly demonstrated that berry polyphenols can inhibit the pancreatic lipase activity *in vitro*,[38] as well as the ability of phenolic extracts of maqui to reduce adipogenesis and lipid accumulation in 3T3-L1 adipocytes.[8] Therefore, maqui berries have demonstrated to be potent inhibitors of pancreatic lipase *in vitro*, with potential to become a natural alternative for the obesity treatment through diet, although further in vivo research is guaranteed.

Sensory evaluation

A preliminary paired comparison test was carried out to evaluate whether trained panellists were able to note organoleptic differences in colour, taste and aroma between the blends made using lemon and the control drinks with citric acid. Results showed significant differences in at least one parameter (colour, taste or aroma) for all beverages pairs ($P < 0.01$) except for LM and AM, with differences attributed to variations in the colour ($P < 0.01$) and aroma ($P < 0.01$) using lemon mixtures with respect to the citric acid controls (data not shown), probably own to the turbidity of lemon juice, which may affect visual appearance whereas its volatile compounds may affect the aromatic profile.[39] In contrast, the sourness:sweetness ratio did not reveal significant differences ($P < 0.01$) between mixtures and controls, as it was expected since sugars and acidity levels were balanced at the study design (data not shown).

Afterwards, descriptive tests carried out with the panellists and showed significant differences among mixtures with lemon for all the sensory properties assessed (Table 4). Colour was significantly differentiated in all beverages containing berry juices, being LM darker than the rest. The presence of anthocyanins in the berry isotonic drinks may account for the observed differences.[27,40,41] The addition of berries to the drinks intensified the aroma of the samples whereas the sourness:sweetness ratio was altered according to added berry (açai and maqui high ratio and blackthorn decreased this parameter, with respect to lemon control) (Table 4). Moreover, when the trained panel was asked for the overall liking (hedonist test) of the beverages, it pointed out that the quality of all beverages was good and satisfactory without any notable differences based on the type of berry used.

Table 4. Sensory evaluation of the isotonic drinks by a trained panel

Drink	Colour	Aroma	Sourness:sweetness ratio	Overall
L	4.68 ± 0.48^{a}	4.27 ± 0.88^{a}	2.59 ± 0.68^{b}	2.86 ± 0.77^{b}
LA	2.82 ± 0.50^{b}	3.09 ± 1.15^{b}	3.32 ± 0.84^{a}	3.83 ± 0.99^{a}
LB	3.20 ± 0.50^{b}	3.73 ± 0.70^{ab}	1.91 ± 0.63^{c}	3.38 ± 0.59^{ab}
LM	1.66 ± 0.52^{c}	3.64 ± 0.79^{ab}	3.23 ± 0.87^{a}	3.00 ± 0.98^{b}
ANOVA *P*-value	***	**	***	**

Juices are scored for sensory parameters on a scale of 1 to 5, where 1 = extremely dark colour, extremely intense aroma, extremely high sourness:sweetness ratio, extremely intense astringency, extremely pleasant; 2 = intense; 3 = moderate (regular colour, aroma, sourness:sweetness ratio, astringency and overall); 4 = slight; 5 = extremely light colour, extremely slight aroma, extremely low sourness:sweetness ratio (higher sweetness than sourness), extremely slight astringency, extremely unpleasant.
Values are mean ± SD.
Different letters means significantly different at $P < 0.05$ according to the Tukey HSD multiple range test.
Significant at $P < 0.01$ (**), and $P < 0.001$ (***).
L, isotonic lemon juice drink; LM, isotonic drink with lemon juice plus maqui berry; LA, isotonic drink with lemon juice plus açaí berry; and LB, isotonic drink with lemon juice plus blackthorn berry.

CONCLUSIONS

New isotonic beverages were evaluated for their quality parameters, sensorial attributes and biological characteristics over 70 days of shelf-life period, and displayed an attractive red colour, due to their anthocyanin contents provided by berries. Maqui isotonic blends were the most active in all antioxidant assays and in the lipase inhibitory assay, and showed the highest TPC by Folin–Ciocalteau reagent, as a result of its higher content in total anthocyanins. Berry mixtures were also the most potent inhibitors of α-glucosidase between all samples. All the studied parameters remained quite stable during preservation, in general, and the new isotonic drinks can be useful to equilibrate redox balance in acute and intense exercise, and support weight loss programmes, avoiding the triglyceride absorption and hyperglycaemia involved in obesity and diabetes mellitus, respectively. Further research in the evaluation of their *in vivo* biological activity and bioavailability is necessary to verify their beneficial effects for sports, nutrition, and health.

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5. PUBLICATION 8 AND PENDING PUBLICATION RESULT: NOVEL MAQUI LIQUOR FOLLOWING THE TRADITIONAL PACHARÁN PROCESSING, AS BERRY ALTERNATIVE PRODUCT

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Maqui berry vs. Sloe berry – Liquor-based beverage for new development

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"Pacharán" is an aniseed liquor-based beverage made with sloe berry (*Prunus spinosa* L.) that has been produced in Northern Spain. On the other hand, maqui berry (*Aristotelia chilensis*) is a common edible berry from Chile, and currently under study because of its multiple beneficial effects on health. The aim of this work was to design a new aniseed liquor-based beverage with maqui berry, as an industrial alternative to a traditional alcoholic product with bioactive berries. The characterization of its composition, compared with the traditional "Pacharán", and its evolution during maceration (6 and 12 months) showed that the new maqui liquor had significantly-higher anthocyanin retention over time. More studies on the organoleptic properties and bioactivity are underway.

Keywords: Maqui, sloe, Pacharán, anthocyanins, chlorogenic acid

Pacharán is a traditional alcoholic beverage obtained by maceration of sloe berries (*Prunus spinosa* L.) in an aqueous ethanol liqueur (25% alcohol by volume, approximately) that contains sugar and essential oils of aniseed (*Pimpinella anisum* L. or *Illicium verum* H.). [1] Currently, industrial production is located in northern Spain, primarily Navarra, where it has been a typical and traditional digestive drink since the 1400s. According to data from the Spanish ministry, Pacharán production was 5000000 L in 2010, approximately, being the second spirit liquor with geographical indication more produced in Spain. [2] Most relevant studies cite sloe as being astringent, diuretic, and purgative, [3] as well as an antioxidant, [4] and anticholinergic. [5] The beverage has an intense and attractive red color, owing to the anthocyanin contribution of the sloe berries during maceration. This group of naturally occurring pigments is of growing interest, not only for technological reasons and due to their organoleptic properties but also because of their potential health-promoting effects, as suggested by the available experimental and epidemiological evidence. [6] Maqui berry (*Aristotelia chilensis*) is a common edible berry from Central and Southern Chile, also a source of natural colorants due to the presence of anthocyanins. Various reports have linked maqui phenolics with high antioxidant capacity, [7] in vitro inhibition of adipogenesis and inflammation, [8] cardioprotection, [9] neuroprotection, [10] and *in vitro* and *in vivo* antidiabetic effects. [7,11] With the perspective of innovation on new products with the potential for health-promoting benefits besides tasty and pleasant experience for the consumer, an aniseed liquor-based beverage with maqui is being designed and studied, in comparison against a traditional Pacharán liquor for its full characterization of phytochemical composition and the evolution of this composition during maceration (6 and 12 months).

Regarding the traditional Pacharán, two cyanidin-glycosides (A7, A9) and two peonidin-glycosides (A10, A11) were identified; these compounds are characteristic of sloe berries (Table 1). [4,5] The new aniseed liquor-based beverage with maqui berry exhibited all the anthocyanins reported for the berry: glycosides and di-glycosides of delphinidin (A1, A2, A5, A6) and cyanidin (A3, A4, A8), according to previous reports. [5,12,13] The levels were significantly higher than for the traditional Pacharán (Table 1).

Table 1: Identification and quantification of different anthocyanins and chlorogenic acid derivatives of sloe and maqui berries, and liquors at 6th and 12th months of maceration

Anthocyanins	Rt	$M\text{-}H^+$ MSn	Liquors (6 months) PS	Liquors (6 months) PM	Liquors (12 months) PS	Liquors (12 months) PM
A1 Dp 3-samb 5-glc	5.8	759, 303	-	8.3 ± 0.1*	-	5.8 ± 0.1
A2 Dp 3,5-diglc	6.6	627, 303	-		-	
A3 Cy 3,5-diglc	11.6	611, 287	-	5.6 ± 0.1*	-	3.7 ± 0.1
A4 Cy 3-samb 5-glc	12.0	743, 287	-		-	
A5 Dp 3-samb	15.7	597, 303	-	3.6 ± 0.3	-	-
A6 Dp 3-glc	16.5	465, 303	-	2.6 ± 0.0	-	0.5 ± 0.1
A7 Cy 3-glc	19.6	449, 287	0.7 ± 0.0	-	-	-
A8 Cy 3-samb	20.2	581, 287	-	1.8 ± 0.0	-	-
A9 Cy 3-rut	21.6	595, 287	1.2 ± 0.0	-	0.1 ± 0.0	-
A10 Peo 3-glc	25.2	463, 301	0.2 ± 0.0	-	-	-
A11 Peo 3-rut	26.8	609, 301	0.8 ± 0.0	-	0.1 ± 0.0	-
TOTAL ANT.			2.9 ± 0.1	21.9 ± 0.4	0.2 ± 0.0	10.0 ± 0.2
Chlorogenic acid derivatives	**Rt**	**$M\text{-}H^-$ MSn**	**Liquors (6 months) PS**	**Liquors (6 months) PM**	**Liquors (12 months) PS**	**Liquors (12 months) PM**
C1 3-caffeoylq	8.9	353, 191	39.6 ± 0.2	-	34.6 ± 0.1	-
C2 3-coumq	13.5	337, 163	3.5 ± 0.1	-	1.3 ± 0.0	-
C3 4-caffeoylq	16.2	353, 173	7.2 ± 0.2	-	7.05	-
C4 3-feruoylq	16.7	367, 193	1.6 ± 0.1	-		-
TOTAL CQA			41.6 ± 0.1	-		-

*A1 and A2, and A3 and A4 coeluted and were quantified together

Cristina García-Viguera is graduated in Pharmacy (1985) from the Faculty of Pharmacy of the University Complutense of Madrid (UCM). Since 1988, she has been following a professional career at the Dep. of Food Science and Technology in the Centro de Edafología y Biología Aplicada del Segura (CEBAS), of the Spanish Research Council (CSIC) where she developed different HPLC techniques for juices, jams and honey analysis. In the year of 1991, she obtained her PhD in Chemistry in the University of Murcia (Spain). After her PhD, she worked in different foreigners laboratories. Mainly, in 1992 she joined the Plant Science Dep. at the University of Oxford, where she got familiar with GC-MS propolis analysis. Later on (1993) she worked at the Consumer Science Department of the Institute of Food Research (IFR), at Reading (UK), in wine polypehnolic analysis. In 1994 she returned to CEBAS-CSIC where developed a new and independent area of research in polyphenolic analysis in the field of food chemistry. In 1999 she became permanent staff of the CSIC as a Tenured Scientist, followed by Scientific Researcher (2004), and Research Professor (2009). During these periods, she created a research group (Phytochemistry Lab, within the Group of Food Quality, Safety and Nutrition) that has been scoring position with some international recognition in the field of phytochemicals in food, and namely in: Characterization and quantification; Chemical transformations of phytochemicals (anthocyanins, and other flavonoids, glusoinolates, minerals and vitamin C) resulting from the food industry processes; Develop of new beverages and foods; Influence of breeding on broccoli inflorescences and sprouts chemical composition; Antioxidant and biological properties of plant compounds. She presents in her CV over 120 original articles published in journals indexed SCI, 4 patents transferred to industries, book chapters, invited conferences, running multiple I&DT projects and supervisions of Master and PhD students. Also she is founding partner of AQUAPORINS & INGREDIENTS a CSIC spin-off.

The total and individual contents of the anthocyanins tended to decrease in both liquors during the period of maceration, being almost fully lost in the sixth month of storage, with respect to the anthocyanin content of both fruits. However, the decrease in the anthocyanin content was lower in the maqui liquor at 6 months of maceration (86.04% vs. 92.29%) and after a year (93.64% vs. 99.44%) (Figure 1). The factors that affect the stability of anthocyanins include structure, pH, temperature, copigments, light, self-association, metallic ions, ascorbic acid, oxygen, sugars and their degradation products, proteins, and sulfur dioxide. [14] The type of anthocyanidin, its glycosylation pattern, and its acylation with aromatic and/or aliphatic acids also have an important effect on anthocyanin stability, although the storage temperature is the main factor determining anthocyanin loss. [15] It is noteworthy that the new maqui liquor had higher anthocyanin content and a lower percentage loss over time.

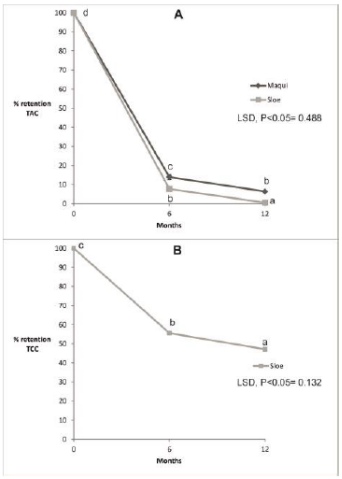

Figure 1: Percentage retention of the total anthocyanins content TAC (A) and total chlorogenic acid derivatives content TCC (B) during the maceration of both berry liquors.

Concerning the chlorogenic acid derivatives, only the Pacharán displayed quantifiable amounts, represented mainly by a large amount of 3-O-caffeoylquinic acid (C1) (Table 1). According to the molecular masses, fragmentation pattern, characteristic spectrum, and reference literature sources,5 3-O-p-coumaroylquinic acid (C2), 4-O-caffeoylquinic acid (C3), and 3-O-feruloylquinic acid (C4) were also identified in significant amounts. It is important to note that there was higher retention of the chlorogenic acid derivatives during maceration, with respect to the total and individual anthocyanins in the Pacharán: 44.41 and 52.90% loss of total chlorogenic acid derivatives after 6 and 12 months of maceration, respectively. This chlorogenic acid stability during storage at 4 and 20 °C has been reported previously in apple juice. [16]

The results show that the anthocyanin levels tended to decrease significantly during maceration, in both liquors, probably due to the effect of the temperature (20 ºC), this loss being less in the new maqui-berry liquor. However, chlorogenic acid derivatives were detected only in the Pacharán, and their retention during maceration was greater with respect to the total and individual anthocyanins in the Pacharán. Some novel bioactive phytochemicals from Pacharán have been identified in this work, and the study of their chemical profile and their evolution during maceration will help to understand how certain phenolics are transferred from fruits to liquors. Studies of their organoleptic properties and bioactivity have commenced.

Experimental

The sloe berries were obtained from Importaciones Samanes S.L (Spain). The maqui berries were provided by Dr. Speisky (INTA-UCHILE) and the aniseed liqueur, called "La Queleña", by Vinos y Licores Renocal S.L. (Spain). The liquors were prepared according to the Spanish regulation of specific denomination "Pacharán Navarro" and its Regulatory Council. [17] The berry fruits were added separately to aniseed liqueur in a proportion of 250 g/L. Then the samples were stored at 20 ºC, in darkness without O2 contact. In addition, the berries were analyzed at the same concentration (250g/L) to obtain the initial values. Both berry liquors (Pacharán and maqui liquor) were stored for 12 months and analyzed after 6 and 12 months of maceration. The analyses were performed in triplicate. The HPLC-DAD-ESI/MSn analyses were carried out with previously reported conditions. [18]

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NOVEL MAQUI LIQUOR FOLLOWING THE TRADITIONAL PACHARÁN PROCESSING

1. INTRODUCTION

Pacharán is a traditional preservative free beverage, obtained by maceration of sloe berries (*Prunus spinosa* L.) in an aqueous ethanol liquor (25% alcohol by volume, approximately) that contains sugar and essential oils of aniseed (*Pimpinella anisum* L. or *Illicium verum* H.) (Fernández-García *et al.*, 1998). Nowadays, industrial production is located in northern Spain, primarily Navarra, where it has been a typical and traditional digestive drink since the 1400s (Regulatory Council of Pacharán from Navarra, 2003). The beverage has an intense and attractive red colour, owing to the anthocyanin contribution of the sloe berries during maceration. Fernández-García *et al*. (1998) identified a total of 18 volatile aroma compounds in commercial pacharán with diethyl malate, trans-anethol and benzaldehyde being the main compounds. Volatile compounds directly affect the sensory quality of fresh and processed fruit products, especially their odour, aroma, and flavour. The concentration of these volatile compounds is generally low, at the level of μg/L, and can be affected by a number of agronomic (variety, climatological conditions, ripening stage, etc.) (Visai & Vanoli, 1997) (Vendramini & Trugo, 2000) and technological (harvest and post-harvest treatments, storage and processing conditions, etc.) factors (Lin *et al.*, 2002).

With the perspective of innovation on new products with the potential for health-promoting benefits besides tasty and pleasant experience for the consumer, an aniseed liquor-based beverage with maqui is being designed and studied, based in the traditional elaboration of *pacharán*. The anthocyanin composition, colour parameters, volatile compounds, and antioxidant capacity were evaluated during maceration (6 months). A final sensory evaluation was carried out using both trained panellists and consumers in order to know the descriptive sensory profile and the consumer acceptance, respectively.

2. MATERIAL AND METHODS

(2.1.) CHEMICALS

The compounds ferric chloride hexahydrate, fluorescein (free acid), 2,2′-azobis(2-methylpropionamidine) dihydrochloride (APPH), 2,4,6-tripyridyl-S′-triazine (TPTZ), sodium dihydrogenphosphate, and disodium hydrogenphosphate were obtained from Sigma-Aldrich (Steinheim, Germany). Meanwhile, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was purchased from Fluka Chemika (Neu-Ulm, Switzerland). Ultrapure water was produced using a Millipore water purification system.

(2.2.) BEVERAGE PREPARATION AND MACERATION

The liquors were processed as described by the Regulatory Council of Pacharán from Navarra (2003). The berry fruits (sloe and maqui) were added separately to aniseed liquor in a proportion of 250 g/L. Then the samples were stored at 20 ^{o}C, in darkness without O2 contact. Both berry liquors, pacharán and maqui liquor, from now on SBP and MBL respectively, were stored for 6 months and analyzed each month and at day 195 (end of maceration). The analyses were performed in triplicate. The fruits were also added to an extractant (50:2:48; water:formic acid:methanol) at the same concentration that in liquors (250 g/L). Then, samples were crushed with an ultraturrax, centrifuged 5 min at 10500 rpm, and filtered through a 0.45-µm PVDF filter (Millex HV13, Millipore, Bedford, MA, USA), in order to know the starting anthocyanin concentration of the fruit.

(2.3.) IDENTIFICATION OF ANTHOCYANINS BY HPLC-DAD-ESI/MS^{N}, AND QUANTIFICATION AND EVOLUTION BY RP-HPLC-DAD

The anthocyanins of both fruits were identified by HPLC-DAD-ESi-MSn analysis. Chromatographic analyses for the identification were carried out on a Luna C18 column (250 x 4.6 mm, 5 mm particle size; Phenomenex, Macclesfield, UK). Water:formic acid (99:1, v/v) and acetonitrile were used as mobile phases A and B, respectively, with a flow rate of 1 mL/min. The linear gradient started with 8% of solvent B, reaching 15% solvent B at 25 min, 22% at 55, and 40% at 60

min, which was maintained up to 70 min. The injection volume was 30 µL. Chromatograms were recorded at 520 nm. The HPLC-DAD-ESI/MSn analyses were carried out in an Agilent HPLC 1100 series equipped with a photodiode array detector and a mass detector in series (Agilent Technologies,Waldbronn, Germany). The equipment consisted of a binary pump (model G1312A), an autosampler (model G1313A), a degasser (model G1322A) and a photodiode array detector (model G1315B). The HPLC system was controlled by ChemStation software (Agilent, version 08.03). The mass detector was an ion trap spectrometer (model G2445A) equipped with an electrospray ionization interface and was controlled by LCMSD software (Agilent, version 4.1). The ionization conditions were adjusted at 350°C and 4 kV for capillary temperature and voltage, respectively. The nebulizer pressure and flow rate of nitrogen were 65.0 psi and 11 L/min, respectively. The full-scan mass covered the range from m/z 100 up to m/z 1200. Collision induced fragmentation experiments were performed in the ion trap using helium as the collision gas, with voltage ramping cycles from 0.3 up to 2 V. Mass spectrometry data were acquired in the positive ionization mode for anthocyanins and negative ionization mode for other flavonoids. MSn was carried out in the automatic mode on the more abundant fragment ion in MS(n-1).

For the quantification all samples were centrifuged for 5 min at 10500 rpm. Each supernatant was filtered through a 0.45-µm PVDF filter (Millex HV13, Millipore, Bedford, MA, USA) before injection into the HPLC system, which was equipped with a Luna C18 column (25 cm × 0.46 cm i.d., 5 µm particle size; Phenomenex, Macclesfield, UK) and a C18 security guard (4.0 x 3.0 mm) cartridge system (Phenomenex, Macclesfield, UK). Water:formic acid (99:5, v/v) and acetonitrile were used as mobile phases A and B, respectively, with a flow rate of 1 mL/min. The linear gradient started with 8% of solvent B, reaching 15% solvent B at 25 min, 22% at 55 and 40% at 60 min, which was maintained up to 70 min. The injection volume was 20 µL. Chromatograms were recorded at 520 nm. Different anthocyanins were characterised by chromatographic comparison with analytical standard (cyanidin 3-*O*-glucoside), and were quantified by the absorbance of their corresponding peak.

(2.4.) ANTIOXIDANT CAPACITY

The free radical scavenging activity was determined using the FRAP (ferric reducing antioxidant power) method adapted to a microscale, according to Mena et al. (Mena *et al.*, 2011). The antioxidant activity was evaluated by measuring the variation in absorbance at 593 nm after 40. The assays were performed using 96-well micro plates (Nunc, Roskilde, Denmark) and an Infinite M200 micro plate reader (Tecan, Grödig, Austria). All the reactions were started by adding 2 µL of the corresponding diluted sample to the well containing the stock solution (250 µL). The final volume of the assay was 252 µL. The antioxidant activity was also determined using the ORAC-FL assay, according to Ou et al. (Ou *et al.*, 2001). The results were expressed as mM Trolox.

(2.5.) COLOR MEASUREMENTS

Solutions were measured in glass cells of 10-mm path length (CT-A21) at 520 nm using a Minolta CM-508i® tristimulus colour spectrophotometer (Osaka, Japan) coupled with a CM-A760 transmittance adapter. CIEL∗, a∗ and b∗ values were calculated using illuminant D65 and a 10◦ observer, according to the CIEL∗ a ∗ b∗ 76 Convention (McLaren, 1980). Data were recorded and processed on a Minolta Software ChromaControl S, PC-based colorimetric data system. Hue angle (H) was calculated from tan−1 (b*/a*) and Chroma (C*) from (a*2 + b*2)1/2. Colour difference were also calculated: ΔE*= [(ΔL*)2 + (Δa*)2 +(Δb*)2]1/2, taking the day 0 of both liquors as a reference. All measurements were done in triplicate, and the mean values reported in each case.

(2.6.) EXTRACTION PROCEDURE OF VOLATILES AROMA COMPOUNDS

Headspace solid phase micro-extraction (HS-SPME) was the method selected to study and semi-quantify the volatile composition of the liquors. For analysis of volatiles, 5 mL of liquor plus 5 mL of ultrapure water were placed in a 50 mL vial with a polypropylene cap and a PTFE/silicone septum; the ratio liquor to headspace was approximately 1:4. A magnetic stirring bar was added, together with NaCl (15 %) to avoid enzymatic reactions and to promote volatiles release.

The vials were equilibrated for 10 min at 40 °C. After this equilibration time, a 50/30 µm DVB/CAR/PDMS fiber was exposed to the sample headspace for 50 min at 40 ºC. The fiber was chosen for its high capacity of trapping fruits volatile compounds.

The extraction conditions were optimized to obtain a volatile profile positively correlated with sensory odour characteristics, as proven by Alonso et al. (Alonso *et al.*, 2009)40 ºC is a temperature which can be close to the body and mouth temperature. Similar extraction procedure has been successfully used in pomegranate wine (Andreu-Sevilla *et al.*, 2013). Extraction experiments were run in triplicate.

After sampling, desorption of the volatile compounds from the fiber coating was carried out in the injection port of the GC-MS during 3 min. The injector temperature was 230 ºC.

(2.7.) CHROMATOGRAPHIC ANALYSES

The isolation and identification of the volatile compounds were performed on a gas chromatograph-mass spectrometer (GC-MS), Shimadzu GC-17A (Shimadzu Corporation, Kyoto, Japan), coupled with a Shimadzu mass spectrometer detector GC-MS QP-5050A. The GC-MS system was equipped with a TRACSIL Meta.X5 column, 95 % dimethyl-polysiloxane and 5 % diphenyl-polysiloxane (Teknokroma S. Coop. C. Ltd, Barcelona, Spain; 60 m x 0.25 mm x 0.25 µm film thickness). Analyses were carried out using helium as carrier gas at a column flow of 0.6 mL min-1 in a split ratio of 1:5 and the following program: a) 80 ºC for 0 min; b) rate of 3.0 ºC min-1 from 80 to 210 ºC and hold for 1 min; c) rate of 25 ºC min-1 from 210 to 300 ºC and hold for 3 min. The temperatures of the injector and detector were 230 and 300 ºC, respectively.

Most of the compounds were identified by using 3 different analytical methods: 1) retention indexes, 2) GC-MS retention times (authentic standards of all compounds reported were used for identification purposes), and 3) mass spectra (authentic chemicals and NIST05 spectral library collection). The volatile studies were conducted in triplicate. The concentration of each compound is expressed as % of the total arbitrary area units as this concentration was named as relative concentration (RC).

(2.8.) SENSORY EVALUATION WITH TRAINED PANEL

Sensory evaluation with trained panel was used to describe both liquors. A panel of 10 panelists, ages 25 to 50 years-old (4 female and 6 male) was trained in descriptive evaluation of alcoholic beverages, including pacharán. All panelists work at Universidad Miguel Hernández de Elche (UMH) and CEBAS-CSIC, and have a wide expertise in sensory evaluation of foods (Andreu-Sevilla *et al.*, 2013; Calín-Sánchez *et al.*, 2013).

Both liquors were assessed using a flavour profile method. Panellists discussed about the main attributes of alcoholic beverages involved in the visual, olfactory and gustative phases, during two preliminary orientation sessions, each lasting 60 min. After these sessions, panellists agreed on their use of appearance and odour/flavour attributes. During these orientation sessions, panellists evaluated different coded samples of both liquors from different manufacturers and/or processed in different ways.

The final test was carried out at UMH facilities (individual booths with controlled illumination, 70-90 fc, and temperature, 20 ± 2 ^{o}C) during three different sessions; samples were evaluated in triplicate. The samples order for each panellist was randomised (AENOR, 1997). Approximately 10 mL of liquor were served into odour-free, disposable 50 mL covered plastic cups (Suministros Monserrate Hernández S.L., Orihuela, Alicante, Spain), at room temperature together with the appropriate questionnaire, one at a time and waiting 5 minutes between samples. Unsalted crackers and water were provided to panellist for palate cleansing between samples. In each questionnaire, panelists were asked, to evaluate the intensity of the following attributes: colour, odour (anise, alcohol, fruity, citric, bitter almond, spice, vanilla, caramel, dried plum and chocolate), basic tastes (sweetness, salty, sour and bitterness), flavours (anise, alcohol, fruity, citric, bitter almond, spice, vanilla, caramel, dried plum and chocolate) and chemical feelings (tongue numb, spicy and oily mouth coating). Panellists used for the evaluation a 11-point scale, where 0 was extremely low intensity, 5 was regular intensity and 10 was extremely high intensity.

(2.9.) CONSUMER STUDY

A sample group of 65 consumers was recruited at the Orihuela campus of

UMH and the facilities of the Research Centre CEBAS-CSIC in Murcia. The group consisted of 36 women and 29 men aged between 18 and 70 years. Consumers lived in the Communities of Valencia and Murcia and more specifically in the provinces of Valencia, Alicante and Murcia; the main requirement for their recruitment was that they consumed alcoholic beverages at least twice a month. The consumer study was carried out at UMH and CEBAS-CSIC during three different sessions. In each session, consumers tested both liquors (each sample was tested three times); the order of samples for each consumer was randomised using William's Latin square design. Approximately 10 mL samples were served at room temperature, one at a time and with a 5 min gap between samples, along with the appropriate questionnaire. Unsalted crackers and water were provided for palate cleansing between samples.

In each questionnaire, consumers were asked, using 9-point hedonic scale (1 = dislike extremely and 9 = like extremely) about their overall liking of the sample and their satisfaction degree for the main pacharán attributes (colour, anise odour, sweetness, bitterness, anise flavour, fruity flavour, dry fruit flavour).

(2.10.) STATISTICAL ANALYSIS

The data here presented are mean values of, at least, 3 replicates and were expressed as the mean ± standard deviation. All the data were subjected to analysis of variance (ANOVA) and a Multiple Range Test (Tukey's test), using IBM SPSS statistics 21 software (SPSS Inc., Chicago, IL, USA). Pearson's correlation analysis was performed to corroborate the relationships between selected parameters.

3. RESULTS AND DISCUSSION

(3.1.) ANTHOCYANINS IDENTIFICATION, QUANTIFICATION AND EVOLUTION DURING MACERATION

The anthocyanin composition of both fruits was identified in a previous works (Barros *et al.*, 2010; Escribano-Bailón *et al.*, 2006; Ganhão *et al.*, 2010;

Schreckinger, Wang, *et al.*, 2010), and confirmed by HPLC-DAD-ESI-MSn analysis (Table 3.1). The initial content of total anthocyanins of the fruits was 11.16 ± 0.40 mg/100mL and 214.43 ± 4.87 mg/100mL for sloe and maqui berries, respectively.

Table 3.1. Anthocyanins identified and quantified (mg/100mL) in traditional pacharán (SBP) and new maqui-liquor (MBL) at the end of maceration (day 195).

Anthocyanins	$[M-H]^+$	MS^n	Rt	Liquor content (mg/100mL)	
				SBP	MBL
Dp 3-*O*-samb-5-*O*-gluc	759	465, 303	8.5	-	19.35 ± 1.72 d
Dp 3,5-*O*-digluc	627	465, 303	10.1	-	15.40 ± 1.39 c
Cy 3,5-*O*-digluc	611	449, 287	14.6	-	7.98 ± 0.91* b
Cy 3-*O*-samb-5-*O*-gluc	743	581, 287	14.6	-	
Dp 3-*O*-samb	597	303	16.2	-	1.07 ± 0.09 a
Dp 3-*O*-gluc	465	303	17.7	-	0.54 ± 0.13 a
Cy 3-*O*-gluc	449	287	21.0	0.19 ± 0.04 ab	1.43 ± 0.18* a
Cy 3-*O*-samb	581	287	21.3	-	
Cy 3-*O*-rut	595	287	23.5	0.37 ± 0.13 b	-
Pe 3-*O*-gluc	463	301	27.8	0.09 ± 0.03 a	-
Pe 3-*O*-rut	609	463. 301	29.7	0.23 ± 0.03 ab	-
LSD, P<0.05				0.066	0.854
TOTAL ANTHOCYANINS				0.87 ± 0.16	45.71 ± 1.08

*Cyanidin 3,5-O-diglucoside and Cyanidin 3-O-sambubioside-5-O-glucoside; and Cyanidin 3-O-glucoside and Cyanidin 3-O-sambubioside (MBL) coeluted and were quantified together. Different letters means significantly different at P < 0.05 according to Tukey HSD Multiple Range Test.

The total and individual content of the anthocyanins had an initial increase in the first month, tended to maintain since day 30 until day 120 (P<0.05), and finally fell by the end of the maceration (Figure 1). Final quantities of anthocyanins were significantly higher in the MBL than in the SBP. The MBL had

a maximum anthocyanin level in day 30 (48.3 % of the initial berry), while SBP had this maximum anthocyanin content in day 60 (27.5 % of the initial berry). Is important to note that anthocyanin degradation of SBP started at day 60, while MBL remained more stable during 3 months (from day 30 until day 120), starting their anthocyanin degradation later (Figure 3.1). Moreover, final MBL also retained higher anthocyanin content from the fruit than SBP (7.77 % and 19.62 %, respectively). Is well known that many factors can affect the stability of anthocyanins, including pH, temperature, copigments, self-association, metallic ions, oxygen, ascorbic acid, sugars and their degradation products, proteins, and sulfur dioxide (Rodríguez-Saona *et al.*, 1999). For that many reasons, anthocyanins passed from the fruits to the liquors in the initial 30-60 days, gradually degraded until the end probably by means of the temperature (20 ^{o}C), presence of sugars from the aniseed liquor, or reactions of copigmentation between anthocyanins and other compounds, such as flavonols (González-Manzano *et al.*, 2009) or phenolic acids (Eiro & Heinonen, 2002), forming other secondary metabolites during storage.

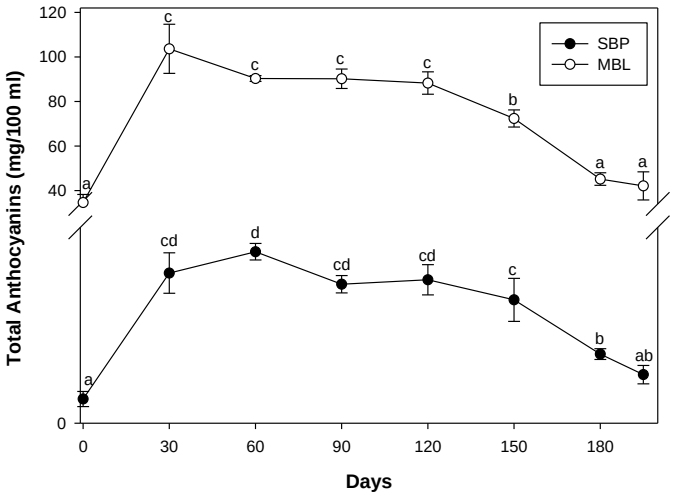

Figure 3.1. Total anthocyanins evolution of traditional pacharán (SBP) and new maqui liquor (MBL) during maceration. Different letters means significantly different at P< 0.05 according to Tukey HSD Multiple Range Test.

(3.2.) ANTIOXIDANT CAPACITY

The antioxidant capacity of MBL and SBP was tested against different reactive species: FRAP, based on the reduction of Fe; and ORAC, based on the ability of peroxyl radical scavenging, providing a more complete evaluation of the antioxidan

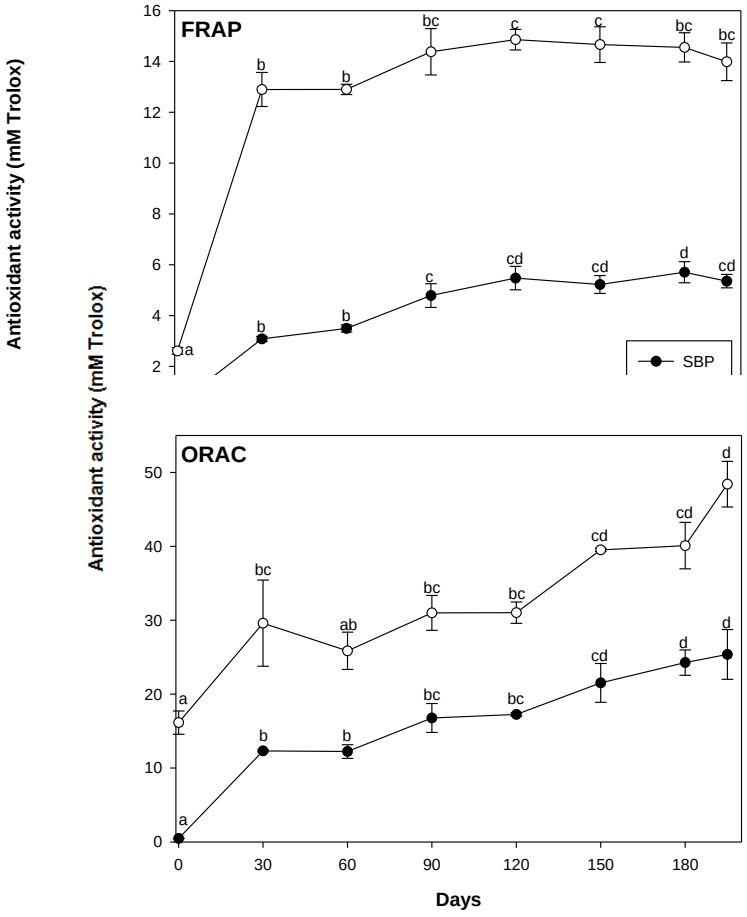

Figure 3.2. Antioxidant activity (FRAP and ORAC) of traditional *pacharán* (SBP) and new maqui liquor (MBL) during maceration. Different letters means significantly different at P< 0.05 according to Tukey HSD Multiple Range Test.

In both assays, new MBL exhibited significant higher antioxidant capacity than the SBP along the maceration. Regarding FRAP assay, both blends displayed a sharp increase in the first 30 days, keeping then a stable increase until the end of the study (5.35 ± 0.26 and 13.99 ± 0.74 mM trolox in final SBP and MBL, respectively). It is important to note that SBP reported higher FRAP scavenging

activity than previously reported for rum, whiskey, and cognac, and the new MBL presented even higher activity than some wines (Pellegrini *et al.*, 2003). The great FRAP scavenging capacity was previously reported for maqui berries, and attributed to their anthocyanin contents (Gironés-Vilaplana *et al.*, 2014), but no significant correlations were found here, being this activity probably also due to other phytochemicals and secondary compounds produced during maceration present in the blends (Pulido *et al.*, 2000).

In the ORAC-Fl assay, an increase throughout the maceration reaching final values of 25.37 ± 3.37 and 48.41 ± 3.08 mM trolox in SBP and MBL, respectively, was observed (Figure 3.2). The bioactive compounds transferred from berries to the liquors, among which anthocyanins can be contributors is well known (H. Wang *et al.*, 1997), but no significant correlation was found in our study between ORAC-Fl values and anthocyanins, since ORAC-Fl values tended to increase and total anthocyanin contents decreased. SBP and new MBL reported higher ORAC-Fl levels than other alcoholic beverages (Escudero-López *et al.*, 2013), and also higher than over 100 different foods, including fruits, vegetables, spices and cereals (Wu *et al.*, 2004).

On the other hand, aniseed liquor without berries did not displayed any activity in these methods (0.09 ± 0.01 and 0.29 ± 0.01 mM trolox for FRAP and ORAC-Fl, respectively), so it can be concluded that the antioxidant capacity of the liquors was provided from the berry contribution. These significant antioxidant activities (FRAP and ORAC-Fl) were previously published for maqui berries (Speisky *et al.*, 2012), and for sloe berries (Dragović-Uzelac *et al.*, 2007) or juice (Fraternale, Giamperi, Bucchini, Sestili, *et al.*, 2009), but is the first time to the best of our knowledge than is reported for alcoholic-based beverages of these berries.

(3.3.) COLOUR CHANGES DURING MACERATION

The newly designed MBL and the SBP with natural anthocyanins of both berries can give an attractive red/dark colour for natural appearance and consumer acceptance, for this reason, colour parameters were determined and studied over 195 days of maceration (Table 3.2).

Table 3.2. Stability of CIEL*a*b* values and colour different values (ΔE∗) of traditional pacharán (SBP) and new maqui-liquor (MBL) during maceration.

SBP									**LSD**
Days	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**195**	**P<0.05**
L*	92.1 g	84.9 f	83.0 e	81.3 d	80.6 cd	79.7 bc	78.8 ab	77.9 a	0.38
a*	0.4 a	6.6 b	7.2 b	8.7 c	9.4 d	10.3 e	10.3 e	11.1 f	0.20
b*	1.0 a	7.7 b	11.2 c	14.6 d	16.6 d	19.3 e	21.4 ef	23.5 f	0.63
Ch	1.1 a	10.2 b	13.3 c	17.0 d	19.0 d	21.9 e	23.8 e	25.9 f	0.59
Hue	70.6 f	49.4 a	57.1 b	59.2 bc	60.4 bcd	61.8 cde	64.2 de	64.8 e	1.22
ΔE*	-	23.9	24.5	25.3	26.1	27.6	29.2	30.6	
MBL									**LSD**
Days	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**195**	**P<0.05**
L*	72.7 b	30.1 a	30.7 a	28.2 a	28.6 a	28.3 a	29.3 a	28.6 a	1.35
a*	23.6 a	58.1 d	55.8 cd	54.2 bc	53.8 bc	52.8 bc	52.1 b	51.6 b	0.89
b*	-3.9 a	32.8 b	52.8 c	48.5 c	49.2 c	48.6 c	50.4 c	49.2 c	3.75
Ch	23.9 a	67.3 b	76.8 c	72.7 bc	72.9 bc	71.8 bc	72.5 bc	71.3 bc	2.13
Hue	350.7 c	29.1 a	43.4 b	41.8 b	42.4 b	42.6 b	44.0 b	43.6 b	2.17
ΔE*	-	66.1	77.5	75.2	75.3	74.7	75.1	74.4	

Different letters means significantly different at P < 0.05 according to Tukey HSD Multiple Range Test.

The CIE*L** values were very different between liquors, being noticeable darker in MBL since day 30, concurring this day with the highest extraction of anthocyanins in the maceration. However, although CIE*L** value remained quite stable over the study in MBL, SBP markedly decreased over time (Table 3.2), as in previous works of colour stability of anthocyanins in juices or anthocyanin-models (Kammerer *et al.*, 2007; Walkowiak-Tomczak & Czapski, 2007). This decrease of lightness may be due to the particles that can pass from the fruit to liquor during the maceration, increasing the turbidity.

With respect to CIE*a** values, MBL reported higher and increasing levels, whereas in SBP a slight increase at day 30 and a steady state could mask the detrimental changes that took place during storage in the anthocyanins (Brenes *et al.*, 2005). In MBL, CIE*a** value showed a steady increase of its characteristic red/dark colour, influenced by anthocyanin transference from the fruits (r = 0.670**, P<0.001), and keept this high CIE*a** value until the end (Table 3.2).

The CIE*b** values exhibited significant increases in the MBL and SBP over

the maceration. This parameter is associated with yellowness and blueness, reporting SBP (characterized by their red/orange colour), higher CIE*b** than CIE*a**, not like MBL with more colour saturation (dark red) and similar final high values for CIE*b** than CIE*a** (Table 3.2).

The Chroma value correlates for the saturation of color and is related to CIE*a** and CIE*b**, so similar patterns were found for SBP and new MBL, showing an increase during all the maceration, and remaining values of MBL quite stable from day 60 to the end of the study. MBL reported higher Chroma values than SBP, as in CIE*a** and CIE*b**, indicative of more colour saturation and influenced by the higher CIE*a** values (dark red) (Table 3.2). Moreover, these Chroma values was correlated with their anthocyanin contents ($r = 0.636^{**}$, $P=0.001$), which was indicative of the influence of the stability of anthocyanins in the colour of new designed liquor (MBL).

Regarding Hue angle, at day 0 both liquors exhibited the highest values, due to the lower values of CIE*a** and CIE*b** (Hue angle= $\tan-1$ (b*/a*)), reporting a significant decrease at day 30, and remaining more stable in maqui liquor over all the maceration (Table 3.2), as in the rest of colour parameters.

Colour difference values ($\Delta E*$) were calculated taking the day 0 of both liquors as a reference, in order to know the changing colour during maceration. In SBP the change was lower than in MBL, but this change was growing until day 195, while in MBL remained quite stable after the initial change at day 30, when this great colour change concurred with the highest value of anthocyanins. Taking into consideration all CIE*L*a*b** parameters, a distinction between the colour evolution of both liquors was noted.

(3.4.) VOLATILE AROMA COMPOUNDS IDENTIFICATION, RELATIVE CONCENTRATION (RC) AND EVOLUTION DURING MACERATION

A total of 36 volatile compounds were isolated from sloe fresh fruits while 35 volatile compounds were found in maqui fresh fruits using HS-SPME (Table 3.3). This extraction technique has been previously used by our research group to study the volatile composition of different fruits, such as mulberries (Calín-Sánchez *et al.*, 2013), and fermented beverages, for instance pomegranate wines (Andreu-Sevilla *et al.*, 2013).

Table 3.3. Volatile aroma compounds identified in fresh sloe, fresh maqui, traditional pacharán (SBP) and new maqui-liquor (MBL) at the end of maceration (195 days).

Compound	RT[¥] (min)	Retention Indexes		Fresh sloe	Fresh maqui	SBP	MBL
		Lit.	Exp.				
Ethyl acetate	5.59	614	612	-	+	-	+
Isoamyl alcohol	6.43	743	749	-	-	+	+
Ethyl isobutyrate	6.75	765	767	-	+	-	-
Isobutyl acetate	6.95	779	779	-	+	-	-
Ethyl Butyrate	7.36	799	803	-	+	-	-
Hexanal	7.43	804	806	+	+	-	-
Ethyl-2-methylbutanoate	8.14	849	842	+	+	-	-
Ethyl isopentoate	8.22	848	846	+	-	-	-
trans-2-Hexenal	8.38	860	854	+	-	-	-
Isoamyl acetate	8.81	876	876	-	+	-	-
2-Hepatanone	9.21	891	897	-	+	-	-
Ethyl pentanoate	9.30	904	901	+	+	-	-
Heptanal	9.44	899	906	+	-	-	-
Methyl hexanoate	9.95	927	923	+	-	-	-
Ethyl tiglate	10.5	938	941	-	+	-	-
6-Methyl-2-heptanone	10.9	955	955	+	-	-	-
Benzaldehyde	11.6	978	979	+	+	+	+
6-Methyl-5-hepten-2-one	11.9	987	990	+	+	-	-
Ethyl hexanoate	12.3	1000	1001	+	+	-	-
(Z,Z)-2,4-Heptadienal	12.6	1006	1009	+	+	-	-
Octanal	12.7	1005	1013	+	+	-	-
Hexyl acetate	12.9	1018	1017	-	+	-	-
(E,E)-2,4-Heptadienal	13.1	1019	1023	+	+	-	-
2-Ethyl-1-hexanol	13.3	1030	1028	-	-	+	+
p-Cymene	13.6	1034	1036	+	+	-	-

Limonene	13.7	1036	1037	+	+	+	+
Benzyl alcohol	13.8	1045	1041	-	-	+	+
1,8-Cineole	14.1	1044	1048	-	+	-	-
Ethyl -2-hexenoate	14.2	1049	1049	+	-	-	-
Phenylacetaldehyde	14.3	1053	1051	+	+	+	+
Diethyl malonate	14.8	1069	1068	-	-	+	+
1-Octanol	14.9	1072	1070	-	-	+	+
2-Octenal	15.0	1062	1070	-	+	-	-
3,5-Octadien-2-one	15.4	1072	1080	+	+	-	-
2-Nonanone	16.1	1096	1100	+	+	-	-
Linalool	16.2	1099	1102	-	-	+	+
Ethyl heptanoate	16.3	1100	1105	-	+	-	-
Nonanal	16.7	1112	1115	+	+	+	+
Methyl octanoate	17.4	1127	1130	+	-	-	-
2,6-Nonadienal	19.1	1158	1167	+	-	-	-
1-Nonanol	19.3	1173	1172	-	-	+	+
(E)-2-Nonenal	19.4	1169	1173	+	-	-	-
Ethyl benzoate	19.6	1172	1179	-	-	+	+
Benzoic acid	19.9	1191	1186	-	-	+	+
Diethyl succinate	20.0	1188	1188	-	+	-	-
Methylphenylacetate	20.2	1186	1193	+	-	-	-
Ethyl octanoate	20.3	1198	1195	+	+	+	+
Estragole	20.9	1200	1208	-	-	+	+
Decanal	21.4	1214	1219	+	+	-	-
β-Cyclocitral	22.5	1234	1243	+	+	-	-
Benzeneacetic acid	22.8	1262	1249	-	-	-	-
trans-Geraniol	23.1	1254	1254	-	-	+	+
Ethylphenylacetate	23.3	1255	1260	+	+	-	-
cis-Anethole	23.4	1262	1262	-	-	+	+
Anys aldehyde	23.9	1250	1272	-	-	+	+
2-Decenal	24.2	1270	1279	+	+	-	-
trans-Anethole	25.5	1307	1306	-	-	+	+
2-Undecanone	25.6	1298	1309	+	+	-	-
(Z,Z)-2,4-Decadienal	25.8	1312	1314	+	-	-	-
Eugenol	28.1	1364	1362	-	-	+	+
Butylhydroxy anisole	29.0	-	1384	-	-	+	+

2-Undecenal	29.1	1376	1386	-	+	-	-
Anisol acetone	29.6	-	1395	-	-	+	+
Tetradecane	29.8	1400	1399	-	-	+	+
Ethyl decanoate	30.3	1405	1411	-	+	-	-
Dodecanal	30.4	1411	1413	-	-	+	+
Neryl acetone	32.9	1460	1470	+	-	-	-
Ethyl cinnamate	33.3	1470	1479	-	-	+	+
β-Ionone	33.8	1485	1490	+	-	+	

*RT, Exp. and Lit. mean Retention Time, Experimental and Literature, respectively.

The volatile aroma compounds found in sloe and maqui fruits can be grouped in 6 chemical groups: a) *esters*: e.g. ethyl acetate, ethyl-2-methylbutanoate, isoamyl acetate; b) *aldehydes*: e.g. hexanal, *trans*-2-hexenal, nonanal; c) *ketones*: e.g. 6-methyl-5-hepten-2-one, 2-nonanone, 2-undecanone; d) *benzene derivates*: benzaldehyde and phenylacetaldehyde; e) *monoterpenes*: limonene and p-cymene and f) *monoterpenoids*: β-cyclocitral.

This is the first scientific evidence of the volatile aroma compounds in both sloe and maqui fruits. Several authors have previously reported the volatile fraction of other fruits belonging to the *Prunus* genus (plums and cherries) as sloe fruits (Prunus spinosa L.). Melgarejo et al. (Melgarejo *et al.*, 2012) and Weng et al. (Wen *et al.*, 2014) found in plums and cherries similar key volatile compounds, such as benzaldehyde, limonene, phenylacetaldehyde, nonanal or 6-methyl-5-hepten-2-one.

The main volatile aroma compounds found in sloe fruits, their RC and their sensory descriptors are: benzaldehyde (RC: 43.2 %; descriptors: bitter almond, fragrant), hexanal (RC: 10.3 %; descriptors: fatty, fruity, green), *trans*-2-hexenal (RC: 8.78 %; descriptors: fruity, green, leafy, apple, vegetable) and nonanal (RC: 5.18 %; descriptors: floral, citrus, orange, rose, fatty). On the other hand, maqui fruits presented the following main volatile compounds, their relative concentration (RC) and their sensory descriptors, respectively: ethyl acetate (RC: 28.5 %; descriptors: balsamic, pineapple), isoamyl acetate (RC: 9.50 %; descriptors: banana, sweet, pear), nonanal (RC: 4.43 %; descriptors: floral, citrus, orange, rose, fatty) and benzaldehyde (RC: 3.08 %; descriptors: bitter almond, fragrant).

Regarding the volatile composition of both liquors (SBP and MBL) at the

end of the maceration, 27 compounds were isolated from both SBP and MBL (Table 3.3). The volatile aroma compounds found in SBP and MBL can be grouped in 11 chemical groups: a) *esters*: e.g. ethyl acetate, diethyl malonate, ethyl benzoate; b) *alcohols*: e.g. isoamyl alcohol, 1-octanol, 1-nonanol; c) *aldehydes*: nonanal, anys aldehyde and dodecanal; d) *phenylpropenes*: estragoles, *cis*-anethole and *trans*-anethole; e) *benzene derivates*: benzaldehyde and phenylacetaldehyde; f) *monoterpenoids*: linalool and trans-geraniol; g) *carboxylic acids*: benzoid acid and benzeneacetic acid; h) *monterpenes*: limonene; i) *phenylpropanoids*: eugenol; j) *ketones*: anisol acetone and k) *linear hydrocarbons*: tetradecane.

The volatile profile of pacharán was previously reported (Fernández-García *et al.*, 1998) and a total of 18 volatile aroma compounds were isolated. In the current study a total of 26 volatiles were found. The results of volatile compounds differ from those of other authors perhaps because of the isolation technique, the type of cultivar or the processing conditions. Fernández-García et al. (1998) employed a continuous liquid-liquid extraction with organic solvents, while the current study was carried out with HS-SPME. This last technique seems to provide better correlations with sensory data and is very useful in the discussion of volatile compounds. Despite these differences, the main volatile compounds were similar in both studies: *cis*-anethole, *trans*-anethole, anisaldehyde, benzyl alcohol, benzaldehyde, ethyl benzoate, estragole, eugenol and linolool.

With regards to the changes with time of the volatile compounds during the maceration process, it is important to mention that there is a clear effect of the maceration with the evolution and final composition of SBP and MBL. It must be highlighted, that three different patterns have been observed. First and due to the low RC in the fresh fruits, several compounds have disappeared along the maceration process, for instance hexanal or *trans*-2-hexenal. On the contrary, some new compounds appeared as result of the maceration; the occurrence of new compounds could be explained by two reasons: (i) some compounds are present in the anise liquor added for the maceration (eugenol or *trans*-anethole) and (ii) the chemical structure of some aldehydes have changed to alcohols due to a reduction reactions; for instance, nonanal decreased its RC while 1-nonanol appeared. Finally and due to the effect of maceration time, the compounds from fresh fruits increased their RC (benzaldehyde) in the final liquors while those from the anise (*trans*-anethole) decreased.

Summarizing, the main volatile compounds of both SBP and MBL due to their relative abundance are: *trans*-anethole (anise, sweet, spicy), benzaldehyde (bitter almond, fragrant), *cis*-anethole (anise, minty, herbaceous) and ethyl benzoate (sweet, spice, musty, dry).

(3.5.) DESCRIPTIVE SENSORY ANALYSIS WITH A TRAINED PANEL

The present study was the first one reporting data on the descriptive analysis of SBP and MBL. The trained panel has more than 100 training hours and wide experience in descriptive sensory analysis (DSA) of fruits and fermented drinks (Andreu-Sevilla *et al.*, 2013; Calín-Sánchez *et al.*, 2013). Sensory results from the trained panel (Figure 3.3) proved that sloe and maqui fruits had appropriate characteristics for beverage liquor processing because of the high intensities of key attributes, such as: (i) colour from 6.5 for SBP to 9.1 for MBL; (ii) sweetness from 6.2 for SPB to 7.3 for MBL; (iii) dry plum odour from 3.9 for SBP to 5.7 for MBL and (iv) caramel flavour from 4.2 for MBL to 5.5 for MBL. On the other hand, some undesirable attributes were scored low, which also contribute to the suitability of both fruits for liquor manufacturing. As examples, astringency ranged from 2.2 for MBL to 2.5 for SBP and sourness got a score of approximately 2.0 for both liquors (Figure 3.3). In summary, descriptive sensory data proved that both liquors under study are appropriate for beverage liquor manufacturing.

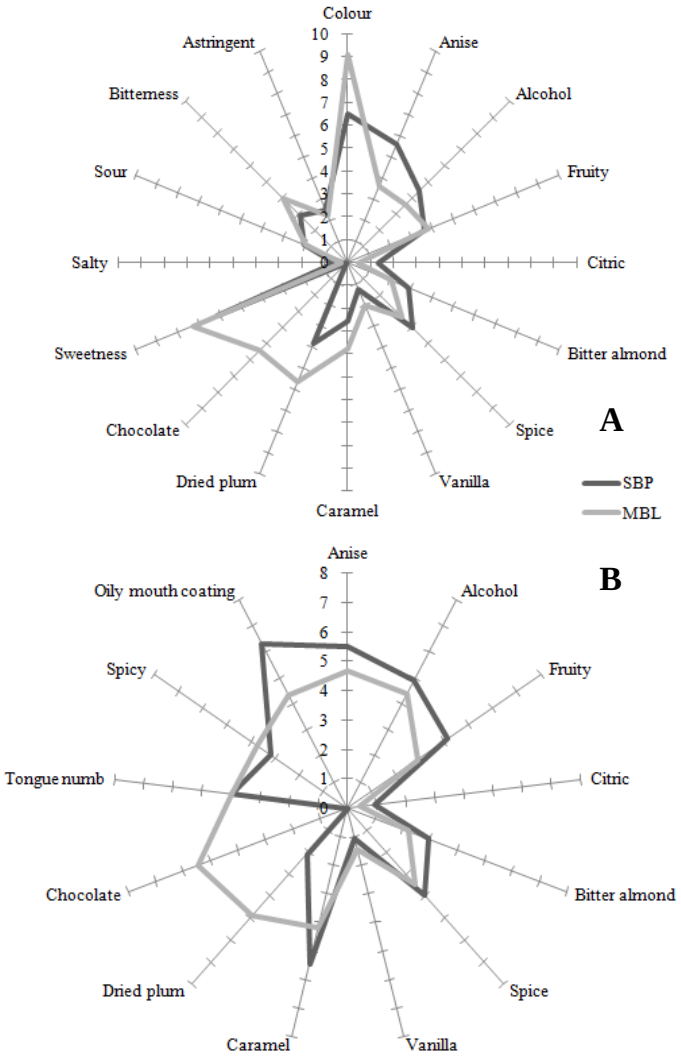

Figure 3.3. Descriptive sensory analysis of traditional pacharán (SBP) and new maqui-liquor (MBL) at the end of maceration (195 days). Odour attributes and basic tastes (A); flavour and chemical feelings (B).

(3.6.) CONSUMER ACCEPTANCE

Table 3.4 showed the satisfaction degree scores of the consumers' panel on the main sensory attributes of traditional *pacharán* (SBP) and new maqui-liquor (MBL) at the end of maceration. The experimental results showed similar acceptance of both liquors with no significant differences except in the case of the colour satisfaction, as expected after the instrumental colour data (Table 2). An overall liking score of approximately 6.0 for both liquors (SBP and MBL) indicates that from an affective point of view, sloe and maqui fruits are highly recommended for beverage liquor processing.

Table 3.4. Satisfaction degree scores of consumer panel from main sensory attributes of traditional *pacharán* (SBP) and new maqui-liquor (MBL) at the end of maceration (195 days).

Attribute	ANOVA[¥]	SBP	MBL
Colour	*	6.0±0.2 a	5.4±0.2 b
Anise odour	N.S.	5.8±0.2	5.6±0.2
Sweetness	N.S.	6.0±0.2	5.9±0.2
Bitterness	N.S.	5.4±0.1	5.7±0.2
Anise flavour	N.S.	5.6±0.2	5.6±0.2
Fruity flavour	N.S.	5.8±0.2	5.7±0.2
Dried fruity flavour	N.S.	5.8±0.2	5.9±0.2
Overall liking	N.S.	6.2±0.2	6.0±0.2

[¥]Values are mean of 65 consumers. Different letters means significantly different at $^{*}P < 0.05$ according to Tukey HSD Multiple Range Test; N.S. not significant differences.

4. CONCLUSIONS

In this study, a new aniseed liquor-based beverage incorporating maqui berry (MBL) was formulated, showing an attractive red colour and higher antioxidant activity than traditional *pacharán*, made with sloe berries (SBP), due to their higher anthocyanin contents and similar volatile composition with 27 volatile aroma compounds in both liquors. Moreover, these anthocyanin levels tended to decrease significantly during maceration in liquors, probably due to the effect of the temperature (20 ^{o}C), copigmentation reactions or sugars interactions, being this loss significantly less accurate in MBL. Descriptive sensory analysis pointed out that all the key and positive attributes (e.g. colour and sweetness) showed high and similar scores in both SBP and MBL; while undesirable attributes (e.g. astringency and sourness) were scored low and also similar in both liquors. Finally, consumers showed a high level of satisfaction for the new maqui liquor (MBL), being similar to that of traditional *pacharán* (SBP), which is the product commercially available for Spanish consumers.

CHAPTER 4. GLOBAL SUMMARY AND GENERAL DISCUSSION

The general aim of this Ph.D. Thesis project was the design of new beverages made of lemon juice and berry fruits with functional properties. In order to fulfil the general objectives, several tasks were accomplished: **(1)** The evaluation of the quality, composition, α-glucosidase and lipase inhibitory activities and the antioxidant capacity of five fruits (native from different countries in Latin America) and of five Spanish *Citrus* fruits, comparing them by different analytical methods (publications 1 and 2). These evaluations helped us to select fruits to be incorporated in new functional beverages. **(2)**Lemon juice enriched with powdered maqui, açaí, sloe, or with chokeberry concentrate were designed (5% *w/v*), and the determination of their phytochemical compounds, together with antioxidant capacity, and their potential to inhibit cholinesterases for applications in adult nutrition and health (publications 3 and 4), were then studied.

(3) Once established that lemon juice enriched with maqui blend was the most valuable regarding bioactive compounds and characteristics, different concentrations of powdered maqui berry were added (2.5% and 5% *w/v*) to lemon juice, and the study of the color parameters, phytochemical composition, and antioxidant capacity, as well as their variations during storage at two different temperatures (4 ^{o}C and 25 ^{o}C), simulating common storage conditions of time and temperature of marketed juices, were carried out (publication 5). Taking into account that organoleptic attributes are essential for the new blends, **(4)** new isotonic drinks made of lemon juice (20 % *v/v* in water) and berries (maqui, açaí and sloe, 5% *w/v*) were also designed, and compared to commercially-available isotonic drinks evaluating the sensorial, and phytochemical characteristics and biological activity over a storage period of 70 days (publications 6 and 7).

(**5**) Finally, a different line of products with an aniseed liquor-based beverage with maqui was also started, based in the traditional elaboration of 'Pacharán', studying the changes over time in the anthocyanin composition, colour parameters, volatile compounds, and antioxidant capacity during maceration (6 months) between the two liquors, and a sensorial evaluation using trained and consumer panels, in order to know the potential for consumer acceptance, was also performed (publication 8 and pending publication).

1. PUBLICATIONS 1 AND 2

Citrus spp. and Latin-American fruits have been used in studies of different foods and drinks with potential application in diet-related diseases and in different health conditions. However, there are insufficient data in the literature (arising from the same assaying procedure and conditions) to allow a comprehensive comparison of the different antioxidant and modulatory activities of enzymes of these polyphenol-rich fruits.

Flavones, flavanones and vitamin C (AA+DHAA) were the main phytochemicals detected in *Citrus* fruits, reporting lemon the highest amounts of flavones.

Among *Citrus* fruits, although grapefruit, lemon and lime performed better in terms of antioxidant capacity and appeared as close correlated with flavanones and vitamin C concentrations, the lemon and lime were the best candidates for antidiabetic and antilypolytic purposes (α-glucosidase and lipase inhibition), also correlated to their vitamin C and flavones content, respectively.

The HPLC-DAD-ESI/MSn analysis of the hydromethanolic extracts of Latin-American fruits revealed a wide range of different phenolic compounds: Anthocyanins and ellagic acid derivatives were only detected in açai and maqui fruits, whilst flavonols, xanthones, and hydroxycinnamic acid derivatives were widely distributed in the Latin-American fruits studied, as previously reported. A point worth mentioning was the higher anthocyanin concentrations in maqui with respect to açaí and other berries found in bibliography, being these amounts responsible of their potential bioactivity.

With respect all the fruits studied, maqui was raised up as the best performer fruit in terms of α-glucosidase and lipase inhibition. Maqui and açaí berries were also the most-interesting fruits in terms of antioxidant capacity according to $ABTS^{+}$, $DPPH^{\bullet}$, FRAP and $O_2^{\bullet -}$ methods, correlated to their high anthocyanin (cyanidin and delphinidin derivatives) contents. Nonetheless, regarding ORAC assay, *Citrus* fruits exhibited higher activity than the Latin-American fruits, in general.

These fruits are clearly valuable sources of phytochemicals for food product development, regarding their applications for nutrition and new dietary options

for the treatment of diseases such as obesity and diabetes.

2. PUBLICATIONS 3 AND 4

The demonstrated nutritional value of certain Latin-American fruits (**1**), and the significant economic activity of *Citrus lemon* in Murcia Region generating fruits for exports and also considerable quantities of non-marketable fruits, prompted us to design new beverages of lemon juice enriched (5% *w/v*) with maqui, açaí, sloe, or chokeberry. Their phytochemical characterization, antioxidant capacity by (DPPH$^{\bullet}$), superoxide ($O_2^{\bullet -}$), hydroxyl radicals ($^{\bullet}$OH), and hypochlorous acid (HOCl) assays, and their potential as inhibitors of cholinesterases (acetilcholinesterase AChE, and butirilcholinesterase BuChE) were investigated. The concentration of fruits powders matched the amount of concentrate added to a citric acid solution (0.18 M, pH=2.46), used as control.

Regarding to the antioxidant capacity, it should be noted that multiple assays gives a broader view to understand the potential for biological activity since these are radicals produced in human cells (superoxide radical, hypochlorous acid and hydroxyl radical). It was also interesting the observation that although samples behaved differently against several reactive species, the novel beverage based on lemon juice and maqui berry (LM) was the most active in the four studied assays, being lemon juice with açaí berry (LA) also great in reactivity against HOCl and $^{\bullet}$OH radicals, and lemon-chokeberry (LAR) against $O_2^{\bullet -}$ and DPPH$^{\bullet}$, respectively. Therefore, lemon-based blends displayed higher antioxidant activities than their controls of citric acid solutions in general, as a result of its higher content in phytochemicals belonging to separate bioactive compounds in the matrix of the new blend. The results suggested that additional factors to the phenolic compounds present were also responsible for their antioxidant potential. The quality composition and the interactions between compounds in the food matrix (i.e. the protection of lemon flavonoids by berry-polyphenols) could be also involved.

Concerning cholinesterases inhibition, all the samples showed activity: the highest potential was found with lemon juice among the controls, being lemon-açaí (LA) the most promising blend, mainly due to the combination with lemon

juice, since direct correlations were found for lemon phytochemicals and AChE and BuChE inhibitory activities.

The fact that these beverages are natural juices enriched with powdered fruits that can be taken regularly, is of interest for developing foods for dietary interventions and nutrition programs for aging adults, probably with a safer profile than drugs and as coadjuvants of treatments.

3. PUBLICATION 5

With the purpose in mind of reaching the market in the near future, it was interesting to know how the phytochemicals of the lemon juice enriched with maqui berry may evolve during storage by simulating the conditions of shelf life. For this purpose, LM was prepared at two different concentrations (2.5 and 5 % *w/v*), and stored for 70 days at two different temperatures (4 ^{o}C and 25 ^{o}C, simulating the refrigerated or shelf storage in the market). Quality and colour parameters (CIELAB) were evaluated during storage, in order to recognize the stability of these essential attributes for the consumer acceptance.

The pH, TA (Total Acidity), and TSS (Total Soluble Solids) values did not change significantly in the mixtures or the control juices over the 70 days of storage at the two studied temperatures. Nevertheless, CIEL*a**b** parameters changed over time. Lightness (CIE*L** value) tended to increase among all the samples and for both temperatures, being more stressed in those samples stored at 25 ^{o}C. Taking into account both redness (CIEL*a**) and yellowness (CIEL*b**), a similar trend was found for all the samples, in spite of the weak statistically differences. With respect to Chroma and Hue angle, an increase was noted for all the samples at all temperatures, apart from a fall in Chroma values of lemon juice control (L), as it was expected considering the strong correlation existing between CIEL*a** and CIEL*b**.

Seven of the anthocyanins previously described plus one new isomer were confirmed by HPLC-DAD-ESI-MSn analysis. The total content of anthocyanins tended to decrease in all samples tested at 25^{o}C, whereas the losses were of less intensity at 4^{o}C, condition that preserved anthocyanins during storage. It is

remarkable that the anthocyanin degradation rate was clearly influenced by the presence of lemon juice in both studied temperatures. Moreover, the rate of decrease in vitamin C content was lower in the mixtures with maqui, with retention of this nutrient until the end of the experiment, demonstrating a protective effect of anthocyanins on vitamin C, also reported by other authors in different models or beverages. However, regardless that losses in total anthocyanin contents over time and that changes in CIEL*a**b** parameters were found, the red coloration remained quite stable until the end of the study, probably as a result of the formation of other coloured-polymers, or copigmentation between anthocyanins and flavonols that can also modify the colour expression by increasing Chroma along with a tone shift towards orange tonalities over time, showing the new beverages a powerful and attractive red colour throughout the study.

Finally, initial high levels of antioxidant capacity and total phenolics content from the new beverages remained quite stable over time, especially at 4°C, and except for the lemon juice control.

4. PUBLICATIONS 6 AND 7

Taking into account that the organoleptic and sensorial acceptability is essential for any new food, new isotonic beverages made of lemon juice (20 % *v/v*) and berries (maqui, açaí, and sloe, 5% *w/v*) were also designed, and completed with the ingredients for the formulation of isotonic beverages (sugar, minerals, etc.). Newly designed isotonic drinks were compared to commercially-available isotonic drinks, and the mineral content, sensorial, phytochemical, and biological characteristics were evaluated over a storage period of 70 days.

The quality parameters did not change in 70 days of storage, such as in the case of blends (publication 6) (**3**), and were within the normal and acceptable values for this kind of drinks. Lemon based isotonic blends also displayed better mineral composition than isotonic commercial beverages, standing out the low Na/K ratio, which may imply positive consequences for health and ionic balance. The presence of minerals like Ca, Fe, P, Mn or Mg in the blends might be also beneficial for a better sporting performance, because the addition of lemon

produced a better mineral profile.

The new isotonic beverages displayed the characteristic flavonoids of each fruit: delphinidin and cyanidin glycosides in maqui-blends, cyanidin glycosides in açaí-blends, and cyanidin and peonidin glycosides in sloe-blends. Regarding the total anthocyanins content, the maqui blends exhibited significant higher quantity than the rest of the samples (42.42 mg/100mL for LM). The total content of anthocyanins tended to decrease over the storage period of 70 days in all samples tested, being these decrease more pronounced in lemon-based drinks with respect to the citric acid controls, probably by the breakdown of anthocyanins by ascorbic acid (as seen in publication 6). Concerning hydroxycinnamic acid derivatives, it is important to emphasize the exceptionally high amount in the sloe samples (AB and LB), due to a large peak of 3-*O*-caffeoylquinic acid, remaining hydroxycinnamic acids practically unchanged over the 70 days of storage. No phytochemical content was present in commercial isotonic drinks, as expected.

Color is crucial for the first response of the consumer to the product. The commercially-available drinks used included artificial colorants in their formulations, like brilliant blue or sunset yellow, to make them more attractive to consumers. The newly-designed isotonic blends with lemon and natural colorants from berries (anthocyanins) had a dark-red color -of natural appearance– very attractive to consumers, displaying statistically-significant differences among the beverages, especially when comparing with the commercial drinks. It is important to note, that regardless of the losses in anthocyanin contents over time, the red coloration of the isotonic blends with berries, remained quite stable during the 70 days of period of study, as a result of the likely formation of other coloured-polymers, or copigmentation between anthocyanins and other flavonoids that appreciably preserved colour and masked the detrimental changes during storage in the anthocyanins.

The new isotonic blends from berries and lemon juice may be useful to regulate some oxidative disorders during intense exercise. The antioxidant capacity of all the samples was measured by different methods ($ABTS^{+}$, $DPPH^{\bullet}$, and superoxide radical ($O_2^{\bullet -}$) scavenging assays), in order to compare the diverse reactivity of the samples in the different assays: aqueous medium ($ABTS^{+}$), methanolic medium ($DPPH^{\bullet}$) and ROS produced in human cells ($O_2^{\bullet -}$). The

commercial isotonic beverages did not display any antioxidant capacity and the new isotonic blends behaved differently against the different reactive species, being the maqui drinks the most active in all the antioxidant assays, likely due to their higher anthocyanin contents. This antioxidant capacity remained quite stable during storage in blends.

With respect to lipase inhibition, the enzyme activity was lower in samples with higher anthocyanin contents displaying sloe and açaí samples significant activity, being the maqui blends particularly effective (41.69 and 43.19 U/L for AM and LM, respectively), and remaining unchanged between days 0 and 70. The commercial isotonic beverages and the citric acid controls did not show any activity. Therefore, maqui berry based drinks were potent inhibitors of pancreatic lipase *in vitro* and so their dietary intake could be used as natural alternative in anti-obesity treatments, although further *in vivo* research is needed.

For the carbohydrate metabolism, the new isotonic blends with berries showed significant α-glucosidase inhibition (around 90%), followed by the citric acid and lemon juice controls (80.3% and 72.6%, respectively), inhibiting the enzyme activity by around 50% of the commercial drinks. This activity remained quite stable during storage. In summary, berry polyphenols from maqui, açaí, and sloe used in functional food developments, such as isotonic beverages, may offer dietary and convenient alternatives to control hyperglycemia in diabetic patients and in subjects who wants to lose weight with the help of physical activity.

Finally, regarding sensory evaluation, colour was significantly differentiated in all beverages containing berry juices, due to the different profiles and content of anthocyanins in the berry-isotonic drinks. The addition of berries to the drinks intensified the aroma of the samples whereas the sourness:sweetness ratio was altered according to added berry (açai and maqui high ratio and sloe decreased this parameter, with respect to lemon control). Furthermore, when trained panel was asked for the overall liking (hedonist test) of the beverages, it pointed out that the quality of all beverages was good and satisfactory without notably differences based on the kind of berry used.

5. PUBLICATION 8 AND PENDING PUBLICATION (9)

With the perspective of innovation on new products for tasty and pleasant experience for the consumer, an aniseed liquor-based beverage with maqui berry (MBL) was designed and studied, based in the traditional elaboration of 'Pacharán' (SBP). In this sense, one preliminary work (*accepted*) (**8**), and a full paper (*under review*) (**9**) were prepared. The anthocyanin composition, colour parameters, volatile compounds, and antioxidant capacity were evaluated during maceration (6 months). Once finished, a final sensory evaluation was carried out using both trained panelists and consumers in order to know the descriptive sensory profile and the potential for consumer acceptance of the new liquor.

The anthocyanin profile of both blends was identified in previous chapters and was confirmed by HPLC-DAD-ESI-MSn analysis, displaying the MBL notably higher anthocyanins quantity than SBP. It is important to emphasize that anthocyanin degradation of SBP started at day 60, while MBL remained more stable during 3 months (from day 30 until day 120), delaying the anthocyanin degradation. Moreover, final MBL also retained higher anthocyanin content from the fruit than SBP (19.62% and 7.77%, respectively). This anthocyanin degradation was probably due to temperature (20^{o}C), presence of sugars from the aniseed liquor, or reactions of copigmentation between anthocyanins and other compounds, such as flavonols and protonated ions from proanthocyanidins fragmentation.

Regarding colour parameters, values were very different between liquors. In this sense, although CIE*L** value remained quite stable over the study in MBL, SBP markedly decreased over time, as in previous works of colour stability of anthocyanins in juices or anthocyanin-models. In MBL, CIE*a** value showed a steady increase of its characteristic red/dark colour, influenced by anthocyanin transference from the fruits ($r= 0.670^{**}$, $P<0.001$), and kept this high CIE*a** value until the end. The CIE*b** values exhibited significant increases in the MBL and SBP over the maceration. This parameter is associated with yellowness and blueness, reporting SBP (characterized by their red/orange color), higher CIE*b** than CIE*a**, not like MBL with more color saturation (dark red) and similar final high values for CIE*b** and CIE*a**. Comparable pattern was followed by liquors for Chroma value, showing an increase during all the maceration, and remaining values of

MBL quite stable from day 60 to the end of the study. Finally, concerning colour difference values (ΔE*), in SBP the change was lower than in MBL, but this change was growing until day 195, while in MBL remained quite stable after the initial change at day 30, when this great colour change concurred with the highest value of anthocyanins.

It is remarkable that this is the first scientific report of the volatile aroma compounds of sloe and maqui berries. A total of 36 volatile compounds were isolated from sloe fresh fruits while 35 volatile compounds were found in maqui berries using HS-SPME being mainly benzaldehyde, hexanal, *trans*-2-hexenal and nonanal in sloe; and ethyl acetate, isoamyl acetate, nonanal, and benzaldehyde in maqui.

The volatile profile of pacharán was previously reported with 18 volatile aroma compounds. In the current study a total of 26 volatiles were found, being the main compounds: *cis*-anethole, *trans*-anethole, anisaldehyde, benzyl alcohol, benzaldehyde, ethyl benzoate, estragole, eugenol and linolool, similar to the previous reports. Summarizing, the main volatile compounds of both SBP and MBL due to their relative abundance are: *trans*-anethole (anise, sweet, spicy), benzaldehyde (bitter almond, fragrant), *cis*-anethole (anise, minty, herbaceous) and ethyl benzoate (sweet, spice, musty, dry). Regarding to the evolution of the volatile compounds during the maceration process, several compounds identified in fresh fruits disappeared along the storage period, new compounds appeared as result of the maceration, and the compounds from fresh fruits increased their relative concentration (benzaldehyde) in the final liquors while those from the anise (*trans*-anethole) decreased.

Sensory results from the trained panel proved that sloe and maqui fruits had appropriate characteristics for beverage liquor processing because of the high intensities of key attributes, in a score of 0 to 10, such as: (a) colour from 6.5 for SBP to 9.1 for MBL; (b) sweetness from 6.2 for SPB to 7.3 for MBL; (c) dry plum odour from 3.9 for SBP to 5.7 for MBL and (d) caramel flavour from 4.2 for SBP to 5.5 for MBL. Furthermore, some undesirable attributes were scored low, which also contribute to the suitability of both fruits for liquor manufacturing.

The consumer evaluation showed similar acceptance of both liquors with no significant differences except in the case of the colour satisfaction, as expected after the instrumental colour data. An overall liking score of approximately 6.0 for

both liquors (SBP and MBL) indicates that from an affective point of view, sloe and maqui fruits are highly recommended for beverage liquor processing. Therefore, the quality characteristics demonstrated that the potential of the new maqui liquor and traditional pacharán was good and satisfactory according to all the parameters under study as well as the descriptive sensory analysis and consumer acceptance.

Once proven the *in vitro* potential of certain berries, mainly maqui berry, demonstrating that blended with lemon juice may preserve better phytochemicals and bioactivities over storage period, and taking into account that as an isotonic drink, the beverages have good sensorial attributes with attractive colour for the consumer acceptance and also good preservation of their phytochemicals and activities over time, it would be interesting to demonstrate their potential health benefits *in vivo*, as well as the bioavailability of their phytochemicals. This new lemon-maqui isotonic drink could be focused not only to sport population, but also as natural alternative for the elderly population with nutritional problems such as obesity, diabetes or cognitive disorders. In this sense, continuing research on their *in vivo* bioactivity and bioavailability, thermal treatments, and microbiological safety, are current challenges to be addressed beyond this Ph.D. Thesis.

6. CONCLUSIONS

The studies conducted in this Doctoral Thesis have led to the following general conclusions:

1. The studied Latin-American and *Citrus* spp. fruits are of great value for nutrition as sources of bioactive compounds for new product developments and diet-related diseases such as obesity and diabetes; and consequently, new interests for food industry regarding functional ingredients would be considered.

2. The development of natural foods rich in inhibitors of cholinesterases, with potential application in preserving cognitive function and managing age-related conditions, and with great antioxidant capacity is possible with beverages made of lemon juice enriched with healthy fruits to facilitate daily intake.

3. New drinks made with lemon juice and maqui berry rich in bioactive phytochemicals demonstrated a high in vitro antioxidant capacity as well as an attractive colour during shelf life. The stability of the composition and the bioactivity of the beverage deserves further attention in functional evaluation and development of new beverages for nutrition and health.

4. Isotonic blends with lemon and anthocyanins-rich berries showed attractive physical and sensorial attributes, and also displayed significantly-higher antioxidant and biological effects than commercial isotonic drinks, which can be useful to equilibrate redox balance in acute and intense exercise, and support weight loss programs avoiding the triglyceride absorption and hyperglycemia.

5. New aniseed liquor with maqui berry can be an acceptable new beverage, comparable to the traditional 'pacharán', with better quality characteristics, higher anthocyanin content and retention, as well as with great scores in sensory analysis and consumer acceptance, offering a new and tasty product for future exploitation.

CAPÍTULO 5: RESUMEN GLOBAL Y DISCUSIÓN GENERAL

El objetivo general de esta tesis doctoral se basó en el diseño de nuevas bebidas con propiedades funcionales hechas de limón con frutos rojos. Para cumplir este objetivo general, se llevaron a cabo otros objetivos específicos: (**1**) La evaluación de la calidad, composición, y la actividad inhibidora de α-glucosidasa y lipasa, así como la actividad antioxidante de cinco frutos (procedentes de diversos países de Latinoamerica) y de cinco frutos cítricos de origen español, comparando entre ellos usando diferente metodología analítica (publicaciones 1 y 2). Estas evaluaciones nos ayudaron a la hora de seleccionar frutos para incorporarlos en nuevas bebidas funcionales. (**2**) Posteriormente, se diseñaron nuevos zumos de limón enriquecidos con liofilizados de maqui, açaí, endrino o con concentrado de aronia (5% *p/v*), y la determinación de sus compuestos fitoquímicos, en conjunto con su capacidad antioxidante y su potencial para inhibir enzimas colinesterasas para aplicaciones en nutrición adulta y salud (publicaciones 3 y 4), fueron estudiadas.

(**3**) Una vez establecido que la mezcla de zumo de limón enriquecida con maqui fue la mejor bebida en cuanto a compuestos bioactivos y otras características, se añadieron diferentes concentraciones de liofilizado de maqui (2.5% y 5% *p/v*) a zumo de limón, y el estudio de parámetros de color, composición fitoquímica y capacidad antioxidante, así como las variaciones de estos parámetros durante la conservación a dos temperaturas diferentes (4ºC y 25ºC), simulando condiciones de tiempo y temperatura de conservación comunes en este tipo de bebidas, fueron llevados a cabo (publicación 5). Teniendo en cuenta que las características organolépticas son esenciales para este tipo de nuevas bebidas, (**4**) nuevas bebidas isotónicas hechas con zumo de limón (20% *v/v* en agua) y frutos rojos (maqui, açaí o endrino, 5% *p/v*) fueron también diseñadas, y comparadas además con otras bebidas isotónicas comerciales evaluando las características sensoriales, fitoquímicas y la actividad biológica durante un periodo de almacenamiento de 70 días (publicaciones 6 y 7).

(**5**) Finalmente, se empezó una línea alternativa de licor anisado con maqui, basado en la elaboración tradicional del 'Pacharán', estudiando los cambios en el tiempo de la composición de antocianos, parámetros de color, compuestos volátiles, y capacidad antioxidante durante la maceración (6 meses), de los dos licores, y llevando también a cabo una evaluación sensorial usando paneles entrenados y de consumidores, para conocer la potencial aceptación del

consumidor (publicación 8 y publicación pendiente).

1. PUBLICACIONES 1 Y 2

Los frutos Latinoamericanos y del genero *Citrus* spp. han sido usados en estudios de diferentes comidas y bebidas con potencial aplicación en enfermedades relacionadas con la dieta y en diferentes condiciones de salud. Sin embargo, no hay suficientes datos en la bibliografía (por lo menos en las mismas condiciones de ensayo y procedimiento) para permitir una comparación exhaustiva de sus diferentes capacidades antioxidantes y actividades moduladoras de enzimas de estos frutos ricos en polifenoles.

Flavonas, flavanonas y vitamina C (AA+DHAA) fueron los principales fitoquímicos detectados en los frutos cítricos, reportando el limón la más alta cantidad de flavonas.

Entre estos frutos cítricos, aunque el pomelo, el limón, y la lima fueron los mejores en términos de capacidad antioxidante correlacionada con sus concentraciones de flavanonas y vitamina C, el limón y la lima fueron los frutos que más actividad antidiabética y antilipolítica (inhibición de α-glucosidasa y lipasa) mostraron, también correlacionada a su cantidad vitamina C y flavonas, respectivamente.

El análisis por HPLC-DAD-ESI/MSn de los extractos hidrometanólicos de los frutos Latinoamericanos reveló un amplio rango de diversos compuestos fenólicos: Antocianos y derivados de ácido elágico fueron detectados solo en açaí y maqui, mientras flavonoles, chantonas, y derivados de ácidos hidroxicinámicos estuvieron ampliamente distribuidos en los frutos Latinoamericanos estudiados, como se ha reportado con anterioridad. Un punto a tener en cuenta es la mayor concentración de antocianos del maqui respecto al açaí y a otros frutos rojos encontrados en bibliografía, siendo estas cantidades responsables de su alta bioactividad.

Con respecto a todos los frutos estudiados, el maqui fue el mejor fruto analizado en términos de inhibición de α-glucosidasa y lipasa. Maqui y açaí fueron también los frutos más interesantes en términos de capacidad antioxidante

de acuerdo con los métodos $ABTS^{+}$, $DPPH^{\bullet}$, FRAP y $O_2^{\bullet -}$, correlacionado a su alto contenido de antocianos (derivados de cianidina y delfinidina). Sin embargo, de acuerdo al método ORAC, los cítricos exhibieron en general mayor actividad que los frutos Latinoamericanos.

Estos frutos son claramente valiosas fuentes de fitoquímicos para el desarrollo de productos alimenticios, con respecto a sus potenciales aplicaciones en nutrición y nuevas opciones dietéticas para el tratamiento de enfermedades tales como la obesidad y la diabetes.

2. PUBLICACIONES 3 Y 4

El demostrado valor nutricional de ciertos frutos Latinoamericanos (**1**), y la significante actividad económica del limón (*Citrus limon*) en la Región de Murcia, generando frutos para exportar y también una cantidad de frutos no aptos para su comercialización, nos llevó a diseñar nuevas bebidas hechas de zumo de limón enriquecidas (5% *p/v*) con maqui, açaí, endrino o aronia. Se determinó su caracterización fitoquímica, su capacidad antioxidante por los ensayos ($DPPH^{\bullet}$), radicales superoxido ($O_2^{\bullet -}$), hidroxilo ($^{\bullet}OH$), y ácido hipocloroso (HOCl), y su potencial como inhibidores de colinesterasas (acetilcolinesterasa AChE, y butirilcolinesterasa BuChE). La concentración empleada de los frutos fue también adicionada a una solución de ácido cítrico (0.18 M, pH=2.46), usado como muestra control.

Con respecto a la capacidad antioxidante, debe resaltarse que múltiples ensayos dan una más amplia visión en orden de comprender mejor el potencial para la actividad biológica, ya que muchos de estos radicales son producidos en las células humanas (radical superóxido, acido hipocloroso y radical hidroxilo). También fue interesante que aunque las muestras reaccionaron de manera distinta dependiendo del radical empleado en cada ensayo, la nueva bebida hecha de zumo de limón con maqui (LM) fue la más activa en los cuatro métodos estudiados, siendo el zumo de limón con açaí (LA) también muy activa frente los radicales HOCl y $^{\bullet}OH$, y limón-aronia (LAR) frente a $O_2^{\bullet -}$ y $DPPH^{\bullet}$, respectivamente. Asimismo, las nuevas bebidas de limón mostraron en general mayor actividad que sus respectivos controles de ácido cítrico, como resultado a

su mayor contenido fitoquímico perteneciente a diferentes fuentes originales en la matriz final de la nueva bebida. Los resultados sugieren que hay más factores adicionales, aparte de los compuestos fenólicos presentes, responsables del potencial antioxidante de las bebidas. La composición e interacciones entre compuestos en la matriz alimentaria (por ejemplo la protección de los flavonoides del limón por los polifenoles de los frutos rojos) pueden verse también involucrados.

De acuerdo a la inhibición de las colinesterasas, todas las muestras mostraron actividad: el zumo de limón exhibió el mayor potencial entre los controles, siendo el limón-açaí (LA) la mejor mezcla, principalmente debido a la combinación con el zumo de limón, ya que se encontraron correlaciones directas entre los fitoquímicos del limón y la actividad inhibidora de AChE y BuChE.

El hecho de que estas bebidas son zumos naturales enriquecidos con frutos liofilizados que pueden ser tomados con regularidad, es de interés para desarrollar productos alimenticios para intervenciones dietéticas y programas nutricionales en personas de edad avanzada, probablemente con un perfil más seguro que drogas y como coadyuvantes de los tratamientos.

3. PUBLICACIÓN 5

Con el propósito en mente de lanzar al mercado en un futuro próximo, es interesante conocer como los fitoquímicos del zumo de limón enriquecido con maqui evolucionan durante el almacenamiento simulando condiciones de vida útil. Para este propósito, LM fue preparada a dos concentraciones diferentes (2.5% y 5% *p/v*), y almacenadas durante 70 días a dos temperaturas diferentes (4ºC y 25ºC, simulando los almacenamientos refrigerados o de estantería del supermercado). Los parámetros de calidad y color (CIELAB) fueron evaluados durante el almacenamiento, en orden de conocer la estabilidad de estos atributos esenciales para la aceptación del consumidor.

El pH, la TA (Acidez total), y el TSS (Solidos solubles totales), no cambiaron de manera significativa en las mezclas o las bebidas control durante los 70 días de almacenamiento en las dos temperaturas estudiadas. Sin embargo, los parámetros

CIEL*a**b** si cambiaron con el tiempo. La luminosidad (valor CIE*L**) tendió a incrementar en todas las muestras en ambas temperaturas, siendo más acentuado en aquellas bebidas almacenadas a 25ºC. Teniendo en cuenta los valores de CIE*a** y CIE*b**, se encontró un patrón similar para todas las muestras, a pesar de débiles diferencias estadísticas. Con respecto al Chroma y al ángulo Hue, se observó un incremento en todas las muestras en ambas temperaturas, excepto una caída de los valores de Chroma en el zumo de limón control (L), como se esperaba considerando la fuerte correlación entre CIE*a** y CIE*b**.

Siete de los antocianos antes descritos, más un isómero fueron confirmados por HPLC-DAD-ESI-MSn. El contenido total de antocianos tendió a disminuir en todas las bebidas a 25ºC, mientras que estas pérdidas fueron de menor intensidad a 4ºC, condición que preservó los antocianos durante el almacenamiento. Es remarcable que la degradación de antocianos fue claramente influenciado por la presencia de zumo de limón en ambas temperaturas. Además, las pérdidas de vitamina C fueron menores en las mezclas con maqui, reteniéndose este nutriente hasta el fin del experimento, y demostrando un efecto protector de los antocianos sobre la vitamina C, antes reportado por otros autores en diferentes modelos o bebidas. Sin embargo, a pesar de las pérdidas en el contenido total de antocianos en el tiempo, y de los cambios encontrados en los parámetros CIEL*a**b**, la coloración roja permaneció bastante estable hasta el final del estudio, probablemente como resultado de la formación de otros polímeros coloreados, o a fenómenos de copigmentación entre antocianos y flavonoles que pueden modificar el color incrementando el Chroma con un cambio de tono hacia tonalidades anaranjadas en el tiempo, mostrando las nuevas bebidas un potente y atractivo color rojo durante todo el estudio.

Finalmente, los altos valores iniciales de capacidad antioxidante y de contenido de fenólicos totales de las nuevas bebidas permanecieron bastante estables durante el almacenamiento, especialmente a 4ºC, y excepto para el zumo de limón control (L).

4. PUBLICACIONES 6 Y 7

Teniendo en cuenta que la aceptabilidad organoléptica y sensorial es

esencial para cualquier nuevo alimento, se diseñaron nuevas bebidas isotónicas hechas de zumo de limón (20% *v/v*) y frutos rojos (maqui, açaí y endrino 5% *p/v*), y completadas con los ingredientes normales para la formulación de bebidas isotónicas (azúcar, sales minerales, etc.). Las nuevas bebidas isotónicas fueron comparadas con otras bebidas isotónicas del mercado, y el contenido de minerales, y las características sensoriales, fitoquímicas y biológicas fueron evaluadas durante un periodo de almacenamiento de 70 días.

Los parámetros de calidad no cambiaron durante los 70 días de almacenamiento, como en caso de las bebidas de la publicación 6 (**3**), y se encontraron dentro de los valores normales y aceptables para este tipo de bebidas. Las nuevas bebidas isotónicas hechas con zumo de limón mostraron mejor composición mineral que las comerciales, destacando la baja proporción Na/K, la cual puede implicar consecuencias positivas en el equilibrio iónico y la salud. La presencia de minerales como Ca, Fe, P, Mn o Mg en las mezclas puede ser beneficiosa para un mejor rendimiento deportivo, ya que la adición de zumo de limón produjo una mejora del perfil de estos minerales.

Las nuevas bebidas isotónicas mostraron los flavonoides característicos de cada fruto: delfinidinas y cianidinas glicosiladas en las bebidas de maqui, cianidinas glicosiladas en las bebidas de açaí, y cianidinas y peonidinas glicosiladas en las bebidas con endrino. Respecto al contenido total de antocianos, las bebidas de maqui mostraron un significativa mayor cantidad que el resto (42.42 mg/100mL para LM). El contenido de antocianos totales tendió a disminuir durante el almacenamiento de 70 días en todas las muestras, siendo esta pérdida más pronunciada en las bebidas con limón con respecto a los controles de ácido cítrico, probablemente debido a la degradación de los antocianos por el ácido ascórbico (como se vio en la publicación 6). Con respecto a los derivados hidroxicinámicos, es importante destacar la excepcionalmente alta cantidad en las muestras con endrino (AB y LB), debido a un enorme pico de ácido 3-*O*-cafeoilquínico, permaneciendo estos derivados hidroxicinámicos prácticamente inalterables durante los 70 días de almacenamiento. Como se esperaba, no se encontraron compuestos fitoquímicos en las bebidas isotónicas comerciales.

El color es crucial en el primer contacto del consumidor con el producto. Las bebidas comerciales incluían en su composición colorantes artificiales en sus formulaciones, como brilliant blue o sunset yellow, para hacerlas más atractivas a

los consumidores. Las nuevas bebidas isotónicas con limón colorantes naturales de los frutos rojos (antocianos) tenían un color rojo oscuro –de apariencia natural- muy atractivo para los consumidores, mostrando diferencias estadísticas significativas entre las bebidas, especialmente cuando se compararon con las comerciales. Es importante resaltar, que a pesar de las pérdidas del contenido de antocianos en el tiempo, la coloración roja de las bebidas con frutos rojos permaneció bastante estable durante los 70 días del periodo de estudio, como resultado probablemente de la formación de otros polímeros coloreados, o fenómenos de copigmentación entre antocianos y otros flavonoides que pueden preservar el color y enmascarar las pérdidas de estos antocianos durante el almacenamiento.

Las nuevas bebidas isotónicas con frutos rojos y zumo de limón pueden ser útiles para regular algunos desordenes oxidativos ocurridos en el ejercicio intenso. La capacidad antioxidante de todas las bebidas fue medida por diferentes métodos ($ABTS^{+}$, $DPPH^{\bullet}$, y radical superóxido radical ($O_2^{\bullet -}$)), para comparar las diversas reactividades de las muestras en distintos ensayos: medio acuoso ($ABTS^{+}$), medio metanólico ($DPPH^{\bullet}$), y ROS producido en células humanas ($O_2^{\bullet -}$). Las bebidas comerciales no mostraron capacidad antioxidante y las nuevas bebidas isotónicas reaccionaron distintamente frente las distintas especies reactivas, siendo las bebidas con maqui las más activas en todos los ensayos, debido a su mayor contenido en antocianos. Esta capacidad antioxidante permaneció estable en todas las bebidas durante el almacenamiento.

Con respecto a la inhibición de la lipasa, la actividad enzimática fue menor en las muestras con mayor cantidad de antocianos, reportando las muestras de açaí y endrino actividad significativa, siendo las mezclas con maqui particularmente efectivas (41.69 y 43.19 U/L para AM y LM, respectivamente), y permaneciendo invariable entre los días 0 y 70. Las bebidas isotónicas comerciales y los controles de solo ácido cítrico no mostraron actividad. Por lo tanto, las bebidas con maqui fueron potentes inhibidores de la lipasa *in vitro* y su ingesta diaria puede utilizarse como alternativa natural en tratamientos anti-obesidad, aunque más investigación *in vivo* debería llevarse a cabo.

Respecto el metabolismo de carbohidratos, las nuevas bebidas isotónicas con frutos rojos mostraron significantes inhibiciones de α-glucosidasa (90% aproximadamente), seguidos por los controles de ácido cítrico y zumo de limón

(80.3% y 72.6%, respectivamente), inhibiendo la encima sobre un 50% las bebidas comerciales. Esta actividad permaneció bastante estable durante el almacenamiento. Resumiendo, los polifenoles de maqui, açaí y endrino usados en el desarrollo de alimentos funcionales, como bebidas isotónicas, pueden ofrecer alternativas dietéticas para controlar la hiperglicemia en pacientes diabéticos y en personas que quieren perder peso con la ayuda de ejercicio físico.

Finalmente, respecto a la evaluación sensorial, el color fue significativamente diferenciado en todas las bebidas que contenían frutos rojos, debido a los distintos perfiles y cantidades de antocianos de estas nuevas bebidas isotónicas. La adición de frutos rojos a las bebidas intensificó el aroma de estas mientras que la proporción acidez:dulzor se vio alterada dependiendo del fruto rojo empleado (açaí y maqui aumentaron la proporción y el endrino la disminuyó, con respecto al limón control). Además, cuando se preguntó sobre la aceptación general de las bebidas al panel entrenado (test hedonista), la calidad de todas las bebidas fue buena y satisfactoria, sin notables diferencias basadas en el tipo de fruto rojo empleado.

5. PUBLICACIÓN 8 Y PENDIENTE

Con la perspectiva de innovar en nuevos productos para experiencias sensoriales sabrosas y agradables para el consumidor, un licor anisado con maqui (MBL) fue diseñado y estudiado, basado en la elaboración tradicional del 'Pacharán' (SBP). En este sentido, se prepararon un trabajo preliminar (*aceptado*) (**8**), y un artículo completo (*en revisión*) (**9**). Se evaluó la composición de antocianos, parámetros de color, compuestos volátiles, y la capacidad antioxidante durante la maceración (6 meses). Una vez finalizada esta, una evaluación sensorial fue llevada a cabo usando paneles entrenados y encuestas de consumidores, en orden de conocer el perfil sensorial descriptivo y la potencial aceptación del consumidor de este nuevo licor.

El perfil de antocianos de ambas bebidas fue identificado en previos capítulos y confirmado en este por HPLC-DAD-ESI-MSn, mostrando MBL una notable mayor cantidad que SBP. Es importante remarcar que la degradación de los antocianos de SBP empezó el día 60, mientras en MBL permaneció más estable

durante 3 meses (de día 30 a 120), retrasándose esta degradación. Además, el licor MBL final retuvo más antocianos del fruto inicial que SBP (19.62% y 7.77% respectivamente). Esta degradación de los antocianos ocurrió probablemente por la temperatura (20ºC), presencia de azucares del anís, o reacciones de copigmentación entre antocianos y otros compuestos, como flavonoles e iones protonados provenientes de la fragmentación de proantocianidinas.

Con respecto a los parámetros de color, los valores fueron muy diferentes entre los licores. En este sentido aunque el valor CIE*L** permaneció bastante estable en MBL durante la maceración, decreciendo en SBP en el tiempo, como en trabajos previos de estabilidad de color en zumos o modelos de antocianos. En MBL, el valor CIE*a** mostro un crecimiento estacionario de su característico color rojo/oscuro, influenciado por la transferencia de antocianos del fruto ($r=0.670^{**}$, $P<0.001$), manteniéndose este alto valor de CIE*a** hasta el final. El parámetro CIE*b** exhibió crecimientos significativos en MBL y SBP en toda la maceración. Este parámetro está asociado con colores amarillos y azules, reportando SBP (característico por su color rojo/naranja), mayor valor CIE*b** que CIE*a**, no como MBL, con mayor saturación de color (rojo oscuro) y con similares valores altos para CIE*b** y CIE*a**. Un patrón comparable fue seguido por los licores para el Chroma, mostrando un incremento durante toda la maceración, y manteniéndose los valores de MBL bastante estables desde el día 60 hasta el final del estudio. Finalmente, de acuerdo con el valor de diferencia de color (AE*), en SBP el cambio fue menor que en MBL, pero este cambio fue creciendo hasta el día 195, mientras que en MBL permaneció estable desde el cambio inicial a día 30, coincidiendo con la mayor cantidad de antocianos.

Es importante destacar que esta es la primera vez que se reporta los compuestos volátiles aromáticos del endrino y del maqui. Un total de 36 compuestos volátiles fueron identificados en el endrino mientras que 35 fueron encontrados en los frutos de maqui usando HS-SPME, siendo principalmente benzaldehído, hexanal, *trans*-2-hexenal y nonanal en endrino; y etil acetato, isoamil acetato, nonanal y benzaldehído en maqui.

El perfil de compuestos volátiles del 'Pacharán' ha sido previamente estudiado, con 18 compuestos aromáticos identificados. En este estudio se encontraron un total de 26 compuestos volátiles, siendo los principales: *cis*-anetol, *trans*-anetol, anisaldehido, benzil alcohol, benzaldehido, etil benzoato, estragol,

eugenol y linolool, similares a trabajos previos. Resumiendo, los principales compuestos volátiles de SBP y MBL de acuerdo con su abundancia relativa fueron: *trans*-anetol (anis, dulce, picante), benzaldehido (almendra aromática, fragante), *cis*-anetol (anis, menta, herbaceo) y etil benzoato (dulce, picante, rancio, seco). De acuerdo a la evolución de estos compuestos durante la maceración, algunos compuestos identificados en los frutos desaparecieron, apareciendo otros como resultado de la maceración, incrementando la concentración relativa de los compuestos de los frutos (benzaldehídos) en el licor final, mientras los del anís decrecieron (*trans*-anetol).

Los resultados sensoriales del panel entrenado demostró que tanto el endrino como el maqui tienen características apropiadas para ser utilizados en la elaboración de licores debido a las altas intensidades de atributos clave como (en escala de 0 a 10): (a) color de 6.5 para SBP hasta 9.1 para MBL; (b) dulzor de 6.2 para SBP hasta 7.3 para MBL; (c) olor a ciruela seca de 3.9 para SBP hasta 5.7 para MBL y (d) flavor de caramelo de 4.2 para SBP hasta 5.5 para MBL. Además, algunos atributos indeseables fueron puntuados bajos, lo cual contribuyó a la idoneidad de ambos frutos para la industria de licores.

La evaluación de consumidores mostró similar aceptación en ambos licores, sin diferencias significativas excepto en el caso de la satisfacción del color, como se esperaba después de los datos instrumentales. Una puntuación de aceptación general aproximadamente 6.0 para ambos licores indicó que desde un punto de vista afectivo, los frutos de endrino y maqui son altamente recomendados el procesado de licores. Por lo tanto, estos resultados demostraron que el potencial del licor de maqui y del pacharán tradicional fue bueno y satisfactorio de acuerdo a todos los parámetros estudiados, así como el sensorial descriptivo y la aceptación del consumidor.

Una vez demostrado el potencial *in vitro* de ciertos frutos rojos, principalmente del maqui, demostrándose que mezclado con el zumo de limón puede preservar mejor sus compuestos fitoquímicos y las bioactividades durante el periodo de conservación, y teniendo en cuenta que como bebida isotónica tiene buenos atributos sensoriales con color atractivo para la aceptación del consumidor y también buena conservación de sus fitoquímicos y actividades durante el almacenamiento, sería interesante demostrar *in vivo* sus potenciales efectos beneficiosos en la salud, así como la biodisponibilidad de sus compuestos

bioactivos. Esta nueva bebida isotónica de limón con maqui podría enfocarse no solo para la población deportista, sino también como alternativa natural para gente adulta con problemas nutricionales como obesidad, diabetes o desordenes cognitivos. En este sentido, se está llevando a cabo investigación más allá de esta Tesis Doctoral acerca de su bioactividad *in vivo*, biodisponibilidad, tratamientos térmicos, y seguridad microbiológica.

6. CONCLUSIONES

Los estudios llevados a cabo en esta Tesis Doctoral han conducido a las siguientes conclusiones:

1. Los frutos Latinoamericanos y cítricos son de gran valor para la nutrición como fuentes de compuestos bioactivos para el desarrollo de nuevos productos y enfermedades relacionadas con la dieta como obesidad o diabetes; y en consecuencia pueden ser considerados de interés para la industria alimentaria como ingredientes funcionales.

2. El desarrollo de alimentos naturales ricos en inhibidores de la colinesterasas, con posibles aplicaciones en la preservación de la función cognitiva y la gestión de enfermedades relacionadas con la edad, y con gran capacidad antioxidante es posible con bebidas a base de zumo de limón enriquecido con frutas saludables, facilitando la ingesta diaria.

3. Nuevas bebidas hechas con zumo de limón y con frutos de maqui ricos en fitoquímicos bioactivos demostraron una alta capacidad antioxidante *in vitro*, así como un color atractivo durante la vida útil. La estabilidad de la composición y la bioactividad de la bebida merece una especial atención en la evaluación y el desarrollo de nuevas bebidas para la nutrición y la salud.

4. Nuevas bebidas isotónicas mezclas de zumo de limón y bayas ricas en antocianos mostraron atributos físicos y sensoriales atractivos, y también mostraron una significativa mayor capacidad antioxidante y actividad biológica que las bebidas isotónicas comerciales, lo que puede ser útil para equilibrar el estrés oxidativo generado en el ejercicio intenso e intenso, y apoyar los programas de pérdida de peso evitando la absorción de triglicéridos y la hiperglicemia.

5. El nuevo licor hecho con maqui puede ser una nueva bebida aceptable, comparable con el tradicional 'pacharán', con mejores características de calidad, mayor cantidad y retención de antocianos, así como con buenas puntuaciones en el análisis sensorial y en la aceptación del consumidor, ofreciendo un nuevo producto sabroso para una posible futura comercialización.

CHAPTER 6. REFERENCES

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