Risk and benefits of beer and nonalcoholic beer moderate consumption on cardiovascular system

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2017

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PROGRAMA DE DOCTORAT NUTRICIÓ I ALIMENTACIÓ

RISK AND BENEFITS OF BEER AND NONALCOHOLIC BEER MODERATE CONSUMPTION ON CARDIOVASCULAR SYSTEM

Memòria presentada per Paola Quifer Rada per optar al títol de doctor per la universitat de Barcelona

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2017

FUNDING SOURCE

Centros de Investigaciones Biomédicas en Red, Fisiopatologia de la Obesidad y Nutrición. CIBEROBN CB06/03 is an initiative of "Instituto de Salud Carlos III"

The European Foundation for Alcohol Research.
EA 1117
EA 1324
EA 1517

Generalitat de Catalunya.
2014 SGR 773
Predoctoral fellowship FI-DGR 2014

Ministerio de Economia, Indútsria y Competitividad.
AGL2013-49083-C3-1-R

Centro de información Cerveza y Salud.
Manuel de la Oya fellowship

University of Barcelona

Bosch I Gimpera Foundation

ABBREVIATIONS:

8PN 8-prenylnaringenin

ADP adenosine diphosphate

APCI atmospheric-pressure chemical ionization

ApoA1 Apolipoprotein A1

CDV Cardiovascular diseases

cGMP cyclic guanylyl monophosphate

CHD Coronary Heart disease

CID collision-induced dissociation

COX cyclooxygenase

eNOS endothelial nitric oxide synthase

ESI electrospray ionization

ET-1 endothelin 1

FDR false discovery rate

FFQ Food-frequency questionnaires

GPIIb/IIIa integrin complex glycoprotein IIb/IIIa

HDL-chl high density lipoprotein cholesterol

HILIC Hydrophilic-Interaction Chromatography

HPLC High performance liquid chromatography

HRMS high-resolution mass spectrometry

ICAM-1 intracellular adhesion molecule 1

IT ion trap

IX isoxanthohumol

LC liquid-chromatography

LC-MS liquid-chromatography coupled to mass spectrometry

LDL-chl low density lipoprotein cholesterol

LOD limits of detection

LOQ limits of quantification

LOX lysyl oxidase

LRMS low resolution mass spectrometry

m/z mass-to-charge ratio

MAPK mitogen-activated protein kinase

MS Mass spectrometry

NF-κβ nuclear factor kappa beta

NO nitric oxide

PLS-DA Partial least square-discriminant analysis

PREDIMED PREvención con DIeta MEDiterranea study

QIT quadrupole ion trap

QqQ triple quadrupole

RP LC reverse phase liquid chromatography

RP reverse phase

SPE solid phase extraction

TNFα like tumor necrosis factor alpha

TOF Time of flight

TxA_2 thromboxane A_2

VCAM-1 vascular cell adhesion molecule 1

XN xanthohumol

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1. ABSTRACT

According to the World Health Organization[1], cardiovascular diseases are the principal cause of death in the world. In 2012, 17.5 millions of people died for cardiovascular diseases, which represents a 31% among all registered deaths. The major causes of cardiovascular diseases are age, sex, smoking, hypertension, overweight and obesity, type-II diabetes, family history of cardiovascular diseases, sedentary lifestyle and diet. Although some of these risk factors are not modifiable, most of the risk factors depend on the lifestyle and habits. The incidence of cardiovascular diseases has not decreased significantly in recent years, thus it is necessary to persist in promoting primary prevention.

Beer is by far the most widely consumed alcoholic beverage in the world. Moderate alcohol consumption in general has been inversely associated with incident cardiovascular disease in observational studies. However, it has been suggested that fermented beverages such as red wine and beer may confer greater protective effects than spirits owing to their higher polyphenolic content. Polyphenols are secondary plant metabolites that are commonly found in fruits, vegetables, legumes, cocoa, tea and fermented beverages[2,3]. Polyphenols are not essential for short-term health status, however there is increasing evidence that long-term intake may reduce the incidence of chronic diseases such as cancer, type-II diabetes, cardiovascular diseases and neurodegenerative diseases[4,5]. Beer phenolic profile is very diverse, most of the beer polyphenols come from malt, and the rest come from hops[6] used during brewing. However, although hops polyphenols in beer are found in low concentrations, they are very specific to beer since they are not found in other food matrices.

In this work, we aim to evaluate whether regular and moderate beer and non-alcoholic beer consumption reduces cardiovascular disease risk. To achieve this goal, we evaluated the effects of ethanol and the phenolic compounds of beer on classical and novel cardiovascular risk factors in participants at high cardiovascular risk. Moreover, we used the PREDIMED study, a large, multicenter, randomized, parallel group and controlled trial, to perform a prospective study aimed at assessing differential associations of wine, beer and spirit consumption with all-cause mortality and incidence of cardiovascular events.

Nutritional biomarkers are indicators of dietary exposure and are important in nutritional epidemiology since this science relies on accurate dietary information to investigate associations between diet and disease risk. Most techniques used in assessing the diet followed by individuals are based on self-reporting, which is liable to systematic bias by factors such as age, gender, social desirability and approval[7,8]. Nutritional biomarkers are very useful in clinical trials for

monitoring compliance with administered interventions. In order to evaluate beer and non-alcohol beer effect on cardiovascular system, a biomarker of beer consumption was needed to measure compliance of volunteers more objectively in clinical studies. Thus, one of the main objectives of this thesis was also to evaluate prenylflavonoids (hops polyphenols found in beer) as biomarkers of beer consumption and asses its effectivity in different clinical trials.

2. HYPOTHESIS AND AIMS

HYPOTHESIS:

In this thesis, two major hypothesis are evaluated:

-Prenylflavonoids from beer may be a specific and accurate biomarker of beer and non-alcoholic beer consumption.

-Moderate and regular beer and non-alcoholic beer consumption may reduce cardiovascular risk.

AIMS:

1. To characterize the polyphenolic profile of beer using high-resolution mass spectrometry.
2. To develop and validate a fast, sensitive, and specific method to quantify xanthohumol, isoxanthohumol and 8-prenylnaringenin in urine using HPLC-MS/MS.
3. To test and validate the potential use of xanthohumol, isoxanthohumol and 8-prenylnaringenin as biomarkers of beer consumption in controlled clinical trials and in free-living population.
4. To evaluate possible metabolic changes after the daily moderate consumption of beer and non-alcoholic beer in male participants at high cardiovascular risk.
5. To evaluate the effects of ethanol and the phenolic compounds of beer on classical and novel cardiovascular risk factors.
6. To evaluate associations of moderate intake of wine, beer and spirits with all-cause and specific mortality
7. To assess the associations of moderate intake of wine, beer and spirits with incidence of cardiovascular events.

3. INTRODUCTION

3.1. Polyphenols

3.1.1. Introduction to polyphenols

The human diet consists largely of plant-derived products, such as vegetables and fruits. Polyphenols are secondary plant metabolites that are commonly found in fruits, vegetables, legumes, cocoa, tea and fermented beverages[2,3].

Polyphenols are structurally very diverse, since more than 8000 structures have been reported[9,10], and are usually classified into two major types: flavonoid and non-flavonoid phenolics. Flavonoid phenolics share a basic skeleton consisting of a biphenylpropane (C_6-C_3-C_6) in which two benzene rings (A and B) are linked through a heterocyclic pyrone C ring. This group includes anthocyanidins, chalcones, flavones, flavonones, flavanols, flavanones and condensed tannins. In contrast, nonflavonoid phenolics include a more heterogeneous group of compounds ranging from the simplest of the class such as benzoic acids (C_6-C_1) to more complex compounds such as stilbenes and lignans. In nature, phenolic compounds are usually found conjugated to sugars and organic acids. The principal chemical structures of polyphenols are shown in **Figure 1.**

Polyphenols are also responsible of some sensory characteristics of food such as color, flavor, bitterness, astringency or aroma. For example, the bitterness of olive oil is mostly caused by oleuropein aglycone and other secoiridoids[11]. Condensed tannins and hydrolysable tannins contribute to the astringency of certain fruits and related beverages, such as grape and wine[12]. Anthocyanidins are responsible for the red, purple and blue colors in fruits and vegetables like berries, red cabbage and black grapes. However, some phenols, like hydroxycinnamic acids, do not contribute to the organoleptic characteristics of the food directly, but if they undergo oxidation, they can lead to the formation of brown polymers which can negatively affect the appearance of the food.

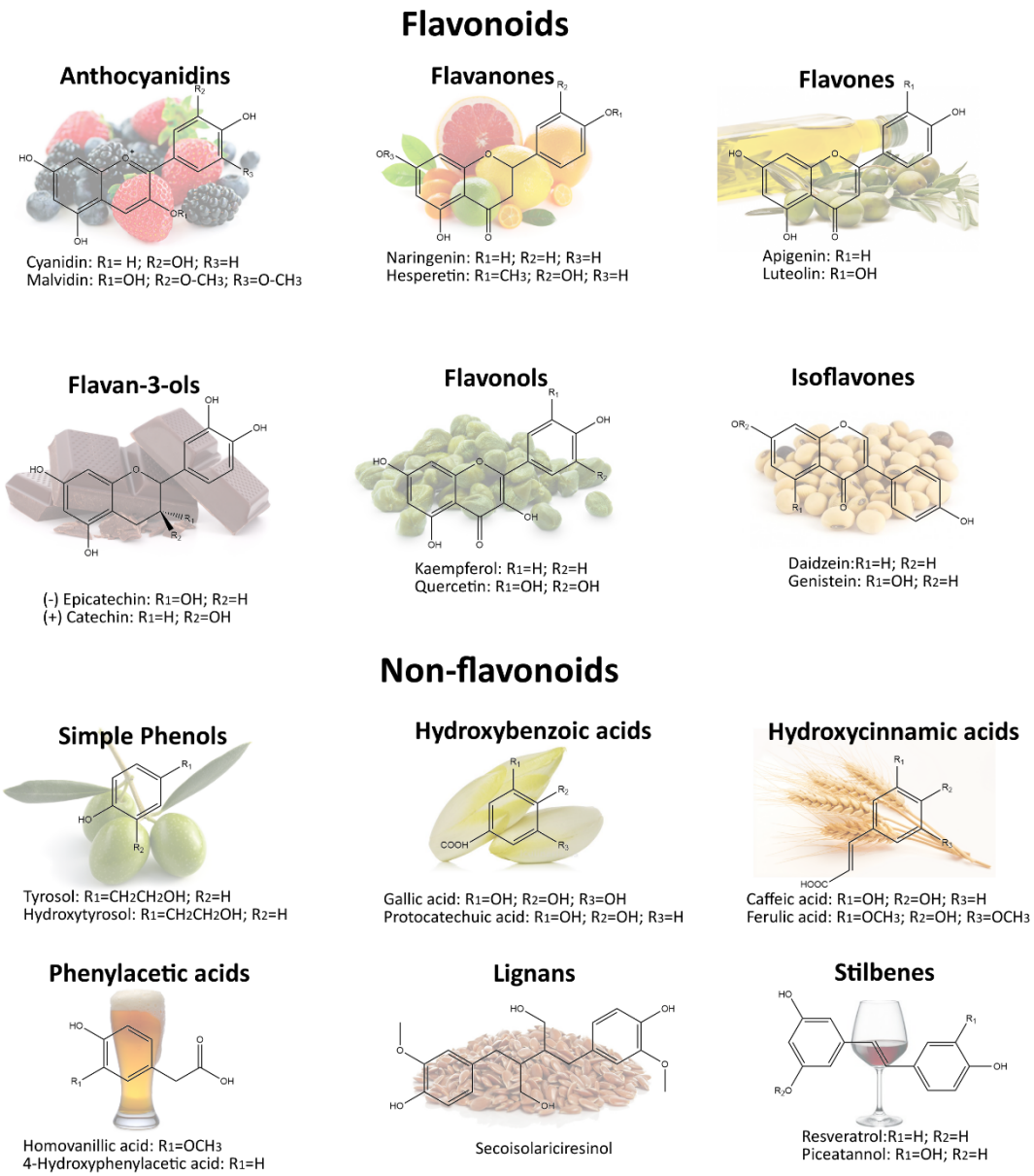

Figure 1. Classification of the main polyphenol's classes along with examples and their food source.

3.1.2. Analytical techniques to identify and quantify polyphenols

The identification and quantification of polyphenols in food and biological samples is a difficult task due to the wide variation of structures present in nature and the lack of standards commercially available. For the identification and quantification of these compounds several methods have been used such as, spectrophotometric methods, capillary electrophoresis, nuclear magnetic resonance spectroscopy, near infrared spectroscopy and chromatographic techniques like high performance liquid chromatography (HPLC), ultra-high performance liquid chromatography (UHPLC), high speed counter current chromatography, supercritical fluid chromatography, and gas chromatography.

Before sample analysis, it is necessary to obtain clean extracts from the extraction of polyphenols using different methods of extraction like, liquid-liquid extraction, solid-liquid extraction, extraction with supercritical fluid and solid phase extraction.

Among all separative techniques, HPLC has been the most widely used. HPLC allows coupling to different detectors such as ultraviolet, fluorescence, refractive index, light scattering, electrochemical, and mass spectrometry detectors. Nowadays high performance liquid chromatography combined with ultraviolet with photodiode array detection or mass spectrometry is the best analytical tool for the quantification and characterization of phenolic compounds.

All these information about the phenolic extraction and analysis in food and biological matrices is contained in the book chapter “Improved Characterization of Polyphenols Using Liquid Chromatography”. Chapter 14 of the book “Polyphenols in Plants: Isolation, Purification and Extract Preparation” (Elsevier, 2014-ISBN:978-0-12-397934-6)[13]

CHAPTER

14 Improved Characterization of Polyphenols Using Liquid Chromatography

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CHAPTER OUTLINE HEAD

14.1 Introduction

Polyphenols are plant secondary metabolites and the most abundant dietary bioactive compounds. Nowadays, it is estimated that 100,000 to 200,000 secondary metabolites exist (Metcalf, 1987). Despite their extreme variety, polyphenols possess a common carbon skeleton building block: the C6–C3 phenylpropanoid unit. Biosynthesis by this pathway leads to a wide range of plant phenols: cinnamic acids (C6–C3),

Polyphenols in Plants. http://dx.doi.org/10.1016/B978-0-12-397934-6.00014-0

benzoic acids (C6–C1), flavonoids (C6–C3–C6), proanthocyanidins [(C6–C3–C6)*n*], coumarins (C6–C3), stilbenes (C6–C2–C6), lignans (C6–C3–C3–C6) and lignins [(C6–C3)*n*] (Seabra *et al.*, 2006).

An exhaustive identification of polyphenols in food and biological samples is of great interest due to their health-promoting effects. Notably, they have an important protective role against a number of pathological disturbances, such as atherosclerosis, brain dysfunction, and cancer (Ignat *et al.*, 2011). It is well known that the protective effects of polyphenols *in vivo* depend on their accessibility and extractability from food, intestinal absorption, metabolism, final biological action in the human body, and potential interaction with target tissues (Tulipani *et al.*, 2012). Phenolics may also act as antifeedants, contributors to plant pigmentation and protective agents against UV light, amongst other activities (Ignat *et al.*, 2011). Nevertheless, the lack of commercially available standards and the wide range of phenolic structures found in nature make identification of phenolic compounds a complex task.

Polyphenol extraction is a crucial step in the development of an analytical method sensitive enough to determine these substances at low concentrations. Several extraction methods are described in the literature (Ignat *et al.*, 2011), but the most common are liquid–liquid extraction (Baydar *et al.*, 2004; Vallverdú-Queralt *et al.*, 2010), solid–liquid extraction (Martinez-Huelamo *et al.*, 2012; Medina-Remon *et al.*, 2009), and extraction with supercritical fluid (Palenzuela *et al.*, 2004; Palma and Taylor, 1999).

Diverse methods have been reported for the identification and quantification of phenolic compounds (Ignat *et al.*, 2011), including spectrophotometry (Huang *et al.*, 2009; Medina-Remon *et al.*, 2009), capillary electrophoresis (CE) (Herrero-Martinez *et al.*, 2005), nuclear magnetic resonance spectroscopy (NMR) (Slimestad *et al.*, 2008), near-infrared spectroscopy (NIR) (Chen *et al.*, 2009), and chromatographic techniques like high-performance liquid chromatography (HPLC) (Martinez-Huelamo *et al.*, 2012; Vallverdú-Queralt *et al.*, 2010), ultra-high-performance liquid chromatography (UHPLC) (Epriliati *et al.*, 2010; Gruz *et al.*, 2008), high-speed counter-current chromatography (HSCCC) (Cao *et al.*, 2009; Yanagida *et al.*, 2006), supercritical fluid chromatography (SFC) (Kamangerpour *et al.*, 2002), and gas chromatography (GC) (Friedman, 2004; Lu and Foo, 1998), although in this chapter we will focus only on liquid chromatography.

Available HPLC detectors have various limitations. Although low detection limits and good sensitivity are obtained by UV, fluorescence, refractive index, light scattering or electrochemical detectors, the structural information they provide lacks detail. The introduction of methods that combine two or more analytical techniques, such as HPLC-UV coupled with photodiode array detection (HPLC-UV-DAD) and HPLC coupled with mass spectrometry (HPLC-MS), has improved structural elucidation of metabolites (Marston and Hostettmann, 2009).

Nowadays, the best analytical tool to quantify and characterize phenolic compounds is considered to be liquid chromatography coupled with ultraviolet-photodiode array detection (UV-DAD) (Chen *et al.*, 2009; Crozier *et al.*, 1997; Epriliati *et al.*, 2010; Fang *et al.*, 2009; Kerem *et al.*, 2004; Liu *et al.*, 2008; Sakakibara *et al.*,

2003; Sun *et al.*, 2007; Wang *et al.*, 2009) or mass spectrometry (MS) (Cao *et al.*, 2009; Chiva-Blanch *et al.*, 2011; Cimpan and Gocan, 2002; Gruz *et al.*, 2008; Han *et al.*, 2008; Martinez-Huelamo *et al.*, 2012; Sanchez-Rabaneda *et al.*, 2003a; Tsao and Deng, 2004; Tulipani *et al.*, 2012; Urpi-Sarda *et al.*, 2009; Vallverdú-Queralt *et al.*, 2010; Volpi and Bergonzini, 2006).

14.2 Sample preparation

14.2.1 Analyte isolation

Accurate identification and quantification of analytes greatly depends on the extraction step. The lack of a standard extraction procedure, which is due to the variability and complexity of phenolic chemical structures and the matrices in which they are found, has led to the proliferation of multiple extraction techniques and methods (Table 14.1).

Extraction can also ensure a more sensitive determination of phenolic compounds and metabolites found at very low concentrations by eliminating interfering components, especially in biological matrices.

Liquid–liquid (LLE) and solid–liquid extraction (which may be followed by solid-phase extraction (SPE) to purify the extract) are the most widely used techniques. Common extraction solvents are methanol, ethanol, acetone, ethyl acetate, and diethyl ether, containing only a small amount of acid. However, polar phenolic acids such as cinnamic acids cannot be extracted with pure organic solvents, and require alcohol–water or acetone–water mixtures.

Liquid samples are usually centrifuged and/or filtered and then the sample is either directly injected into the separation system or analytes are isolated using LLE or SPE.

Conventional methods such as boiling, heating or refluxing can be used to extract natural phenolic compounds from samples, but polyphenols can be lost due to hydrolysis, ionization and oxidation during the process (Li *et al.*, 2005). In recent years, other techniques have been developed for polyphenol extraction, including ultrasound-assisted, microwave-assisted, supercritical fluid, and high hydrostatic pressure extraction (HHP) (Wang and Weller, 2006).

Supercritical fluid extraction is being increasingly used in food and pharmaceutical industries as it is more environmentally friendly, avoiding the use of large amounts of toxic solvents, as well as being rapid, automatable, and selective (Bleve *et al.*, 2008; Maróstica-Junior *et al.*, 2010). The intrinsic low viscosity and high diffusivity of supercritical CO_2 has permitted faster and more efficient separation, and relatively clean extracts. In addition, the absence of light and air during extraction reduces the degradation of analytes that occur in traditional extraction techniques. Supercritical fluids have solvating powers similar to organic solvents but with higher diffusivity, lower viscosity, and lower surface tension. However, the solvating power of a supercritical fluid needs to be controlled by temperature and pressure, or by adding organic modifiers such as methanol. For example, owing to the polarity of

Table 14.1 Examples of Extraction and Analysis of Polyphenols in Food and Biological Samples

Matrix	Analytes	Extraction	Analysis	LOD (mg/l)	References
Food and Beverage Samples					
Fruits and fruit juices	Phenolic acids, anthocyanins, hydroxybenzoic acids, flavan-3-oles, hydroxycinnamic acids, coumarins, flavanones, flavones, dihydrochalcones, flavonols	SE LLE	HPLC-UV HPLC-ESI-MS	0.03–0.005	Abad-García *et al.*, 2007; Liu *et al.*, 2012; Sakakibara *et al.*, 2003; Xu *et al.*, 2012; Fang *et al.*, 2009
Grapes and grape juices	Anthocyanins, flavanols, flavonols, hydroxycinnamates	SE LLE	HPLC/Q-TOF HPLC-MS/MS HPLC-DAD HPLC-MS	3–0.5	Liang *et al.*, 2011; Muñoz *et al.*, 2008; Xu *et al.*, 2012
Vegetables	Quercetin glycosides, hydroxycinnamic acids, phenolic acids, flavanols, flavonols, flavones	SE SPE	HPLC-UV CE UHPLC-DAD	0.62–0.005	Caridi *et al.*, 2007; Silva *et al.*, 2012
Tea leaves and derived products	Flavanols, hydroxycinnamic acids, phenolic acids, flavones, phenolic terpenes, hydroxybenzoic acids	SE	HPLC-DAD-ESI-MS/MS UHPLC-UV UHPLC-MS/MS	0.048–0.0301	Aura *et al.*, 2002 ; Spáčil *et al.*, 2010; Wang *et al.*, 2008a
Apples	Flavanols, flavonols, hydroxycinnamates, anthocyanins, dihydrochalcones	SE	HPLC-DAD HPLC-MS	3×10^{-7}– 3×10^{-8}	Alonso-Salces *et al.*, 2005; Vrhovsek *et al.*, 2004
Wine	Flavanols, flavonols, phenolic acids, stilbenes, hydroxycinnamates, hydroxybenzoic acids, procyanidyns, cinnamic acids	LLE MEPS Filtration	HPLC-UV-FLD UHPLC-DAD HPLC-UV-DAD	0.54–0.02 0.2–0.01 0.05–0.003	Bétes-Saura *et al.*, 1996; Gonçalves *et al.*, 2013; Rodrí;guez-Delgado *et al.*, 2001
Alcohol-free beer	Flavanols, hydroxycinnamates	SPE	HPLC-UV	0.2–0.01	Garcia *et al.*, 2004

Beans, soy beans and derived products	Flavanols, phenolic acids, hydroxycinnamates, isoflavones	SE	HPLC-UV-DAD HPLC-ESI-MS UHPLC-UV	<0.5	Griffith and Collison, 2001; Ross *et al.*, 2009; Toro-Funes *et al.*, 2012
Cocoa and chocolate	Catechin and epicatechin, procyanidins	LLE SE	HPLC-FLD UHPLC-MS/MS	$0.002–2\times10^{-6}$ 20–9	Machonis *et al.*, 2012; Ortega *et al.*, 2010
Olive oil	Tyrosols and flavonols	LLE	HPLC-ECD	<4	Capannesi *et al.*, 2000
Water	Phenolic acids, flavonols, hydroxycoummarics	SE	HPLC-DAD	0.3–0.1	Liu *et al.*, 2008
Propolis	Flavonoids	LLE	HPLC-MS	<0.0025	Volpi and Bergonzini, 2006
Tomato and derived products	Phenolic acids, flavonols, flavanones, hydroxy-cinnamic acids	LLE	HPLC-MS/MS, HPLC-QTOF, HPLC-Orbitrap	1.7×10^{-5} 3×10^{-7}	Vallverdú-Queralt *et al.*, 2010, 2011a, 2011b; 2011d, e 2012b, c
Biological Samples					
Rat urine and plasma	Isoflavones and its metabolites Puerarin (daidzein-8-C-glucoside) and its metabolites Epicatechin, epigallocatechin and its metabolites	SPE Protein precipitation LLE	HPLC-MS/MS HPLC-ESI-MS/MS HPLC-UV	0.125–0.025	Fang *et al.*, 2002; Fu *et al.*, 2008; Prasain *et al.*, 2004a
Serum	Isoflavones and lignans Procyandin B1	SPE LLE	HPLC-ESI-MS/MS HPLC-MS	$<1\times10^{-10}$	Grace *et al.*, 2003; Sano *et al.*, 2003
Urine	Phenolic acids, Flavanols, flavonols Flavanols, flavonols, phenolic acids and related metabolites	LLE Protein precipitation SPE	HPLC-ESI-MS/MS UHPLC-ESI-MS/MS	1.3×10^{-7}– 1×10^{-10}	Magiera *et al.*, 2012; Martinez-Huelamo *et al.*, 2012; Rios *et al.*, 2003
Plasma	Epicatechin and related metabolites Quercetin Flavanols, flavonols, phenolic acids and related metabolites	SPE	HPLC-UV/Vis-FLD-ECD HPLC-ECD HPLC-ESI-MS/MS	<0.2 2.9×10^{-7}– 5×10^{-9}	Erlund *et al.*, 1999; Martinez-Huelamo *et al.*, 2012; Ottaviani *et al.*, 2012

LLE, liquid–liquid extraction; SE, solid extraction; SPE, solid-phase extraction.

anthocyanins, their extraction by the SC–CO_2 method requires high pressures and the presence of methanol or ethanol (Bleve *et al.*, 2008).

Ultrasound-assisted extraction is an inexpensive, simple, and efficient alternative to conventional extraction techniques (Wang *et al.*, 2008b). This method extracts non-volatile and semi-volatile compounds from the matrix. The ultrasonic process facilitates contact between the sample matrix and extraction solvent. Ultrasonication is often carried out to improve phenolic compound extraction from plants; for example, a study with *Folium eucommiae* (Huang *et al.*, 2009) found it to be more efficient than conventional extraction techniques.

Another promising approach for extracting phenolic compounds is microwave-assisted extraction, which was satisfactorily used to analyze gallic acid, protocatechuic acid, chlorogenic acid and caffeic acid in Eucommia ulmodies (Li *et al.*, 2004). Zhang *et al.* extracted polyphenols from Camellia oleifera fruit hull using microwave-assisted extraction (Zhang *et al.*, 2011), finding the optimal conditions to be a liquid : solid ratio of 15.33 : 1 (ml/g), extraction time of 35 min and extraction temperature of 76°C. The same method has also been used to extract polyphenols from green tea (Nkhili *et al.*, 2009) and found to be more efficient than conventional heating.

Enzymatic release of phenolic compounds is also employed to extract phenolic compounds, for example, from grape pomace (Maier *et al.*, 2008). Another study investigated the ability of three enzymes (Ultraflo L, Viscozyme L, and a-Amylase) to release phenolic compounds from *Ipomoea batatas* L. (sweet potato) stems (Min *et al.*, 2006). Ferulic acid release rate was optimal when Ultraflo L (1.0%) was used, whereas Viscozyme L was the most effective for the release of vanillic acid and vanillin.

Another technique that enhances the extraction of polyphenols is HPP. Studies carried out by Shouqin *et al.* (2005) have demonstrated the benefits of hydrostatic pressure for the extraction of flavanols.

14.2.2 Analyte purification. Solid phase Extraction

SPE is an extraction technique used as a clean-up procedure and a pre-concentration step with crude plant, biological, environmental, food, and pharmaceutical samples (Ho *et al.*, 2012; Kerio *et al.*, 2012; Martinez-Huelamo *et al.*, 2009; Navas, 2012; Olmos-Espejel *et al.*, 2012).

Alkyl-bonded silica or copolymer sorbents are commonly used to extract analytes, reverse-phase sorbents being most chosen for polyphenols. Samples and solvents are usually slightly acidified to prevent ionization of phenolic compounds, which would lead to a weaker analyte retention in the sorbent (Navas, 2012; Vinas *et al.*, 2011).

In a recent study, reverse-phase HLB cartridges were used to extract phenolic compounds and metabolites from the urine and plasma of volunteers who had consumed different types of tomato sauce: without oil, or containing 5% of virgin olive oil or 5% of refined olive oil (Martinez-Huelamo *et al.*, 2012). Urpi-Sardà *et al.* (2009) also used HLB cartridges to extract conjugated phenolics from urine and

plasma after regular consumption of cocoa. SPE is essential when working with LC-MS in order to reduce the matrix effect, especially when analytes are found in low concentrations, as occurs in biological samples. However, HLB cartridges have also been used to determine resveratrol and piceid in beer matrices (Chiva-Blanch *et al.*, 2011).

Mix-mode cation/anion exchange reverse-phase sorbents have also been applied to extract phenolic compounds from biological matrices due to their higher capacity to clean up samples. In a study by Medina-Remon *et al.* (2009), HLB, MCX (mix-mode cation-exchange reverse-phase sorbent) and MAX (mix-mode anion-exchange reverse-phase sorbent) were compared in the extraction of 10 representative polyphenols from urine samples, and the best recoveries were obtained with MAX cartridges. Vallverdú-Queralt *et al.* used MAX cartridges to analyze phenolic compounds of different tomato varieties (Vallverdú-Queralt *et al.*, 2011e) and to distinguish between organic and conventional tomatoes (Vallverdú-Queralt *et al.*, 2012a). In a study carried out to analyze colonic microbial metabolites, MCX was used to extract polyphenols from urine and plasma after regular consumption of cocoa (Urpi-Sarda *et al.*, 2009).

14.3 High performance liquid chromatography (HPLC)

The type of column used to separate phenolics and their glycosides is almost exclusively a reverse-phase C18-bonded silica column ranging from 100 to 300 mm in length and with an internal diameter of 2–4.6 mm (Merken and Beecher, 2000; Stalikas, 2010; Tsao and Deng, 2004; Tulipani *et al.*, 2012), although occasionally C8 columns are used to separate phenolic acids. Columns are maintained from room temperature to 40°C during the analysis but thermostated columns give more repeatable elution times and greater resolution, and allow the backpressure of the LC column to be reduced at high flow rates.

The use of a binary system is essential for the separation of structurally varied phenolic compounds. Gradient elution is usually performed with a solvent A, including an aqueous acidified polar solvent or water-containing buffer, and a solvent B, which can be an organic solvent such as methanol or acetonitrile, pure or acidified (Merken and Beecher, 2000; Tsao and Deng, 2004). The volume of injection ranges from 1 to 100 µl, depending on the internal diameter of the column used (Merken and Beecher, 2000).

14.3.1 Ultraviolet detection

Ultraviolet (UV) is the simplest and most commonly used HPLC detector due to its greater sensitivity, linearity, versatility, and reliability (Wolfender, 2009). The existence of conjugated double and aromatic bonds in phenolic compounds allows them to absorb UV or UV-VIS light (Stalikas, 2010). There are different types of UV detectors: fixed wavelength, multiple wavelength, or photodiode array (DAD)

(Wolfender, 2009), the last one being the most frequently used to detect phenolic compounds.

Polyphenols absorb light at different wavelengths. Flavonoids have two characteristic absorption bands: the first has a maximum in the 240 to 285nm range, corresponding to the A-ring, while the second band has a maximum in the 300 to 550nm range, which is attributed to the substitution pattern and conjugation of the C-ring. Anthocyanins also present two absorption bands, in the regions of 265–275 and 465–560 nm. Flavones, flavonols, and flavonols are detected at 280 and 350 nm. UV spectra of catechins give peaks at 210, 278, and 280 nm. Flavones and flavonols have bands in the ranges of 240–280 nm and 300–380 nm. Flavanones and isoflavones are detected at 280–290 nm and 236–262 nm, respectively (Crozier *et al.*, 1997; Sakakibara *et al.*, 2003). Figure 14.1 shows the UV spectra of representative polyphenols.

UV detection became the preferred detector in LC analysis since it is cheap and robust, especially for food matrices containing high phenol concentrations (Table 14.1). In a study by Bétes-Saura *et al.* (1996) an HPLC coupled with a UV-DAD detector was used to identify and quantify 30 polyphenols in white wines. The column used was a C18 (250 × 4 mm), with 5 µm particle size. Flow rate was set at 1.5 ml/min and gradient elution was performed with glacial acetic acid in water at pH 2.65 (phase A) and 20% solvent A mixed with 80% acetonitrile (phase B). The chromatogram was monitored simultaneously at three wavelengths: 280, 320, and 365 nm. Benzoic acids, tyrosol, flavan-3-ols, and the oligomeric procyanidins were quantified at 280 nm, cinnamic acids and their tartaric esters at 320 nm and flavonols at 365 nm (Caporaso *et al.*, 2011). The method was validated, providing good precision and linearity and low limits of detection, which varied from 0.003 mg/l for cis-caftaric acid to 0.051 mg/l for tyrosol.

Liu *et al.* (2008) developed a method to determine polyphenols in water by HPLC-DAD. The separation of phenolic compounds was carried out in a C18 column (150 × 4.6 mm, 5 µm). Gradient elution was performed using acetic acid/water solution (1:99, v/v) as the aqueous mobile phase and methanol as the organic phase. The photodiode array detector operated between 210 and 400 nm. The method was validated, with recoveries between 83 and 95% and limits of detection ranging from 0.1 to 0.3 mg/l. The developed method allowed the identification and quantification of seven polyphenols (chlorogenic acid, esculetin, caffeic acid, scopoletin, rutin, quercetin hydrate, kaempferol) in tobacco-polluted water.

Another study by Lachman *et al.* (2009) used HPLC-DAD to analyze anthocyanidins in red- and purple-fleshed potatoes from 15 cultivars. Anthocyanidins were determined using a reverse-phase column C18 (4 × 250 mm, 7 µm). Solvent A was aqueous 1% (v/v) phosphoric acid, 10% (v/v) acetic acid, 5% acetonitrile (v/v), and solvent B was 100% HPLC grade acetonitrile at a flow rate of 1 ml/min. The quantification of anthocyanidins was set at $\lambda = 530$ nm. Prior to HPLC analysis, samples were hydrolyzed by acidic hydrolysis. The results showed that individual cultivars differed significantly in the relative proportion of anthocyanidins. However, the most abundant anthocyanidin in red- and purple-fleshed potatoes was petunidin (46.9%),

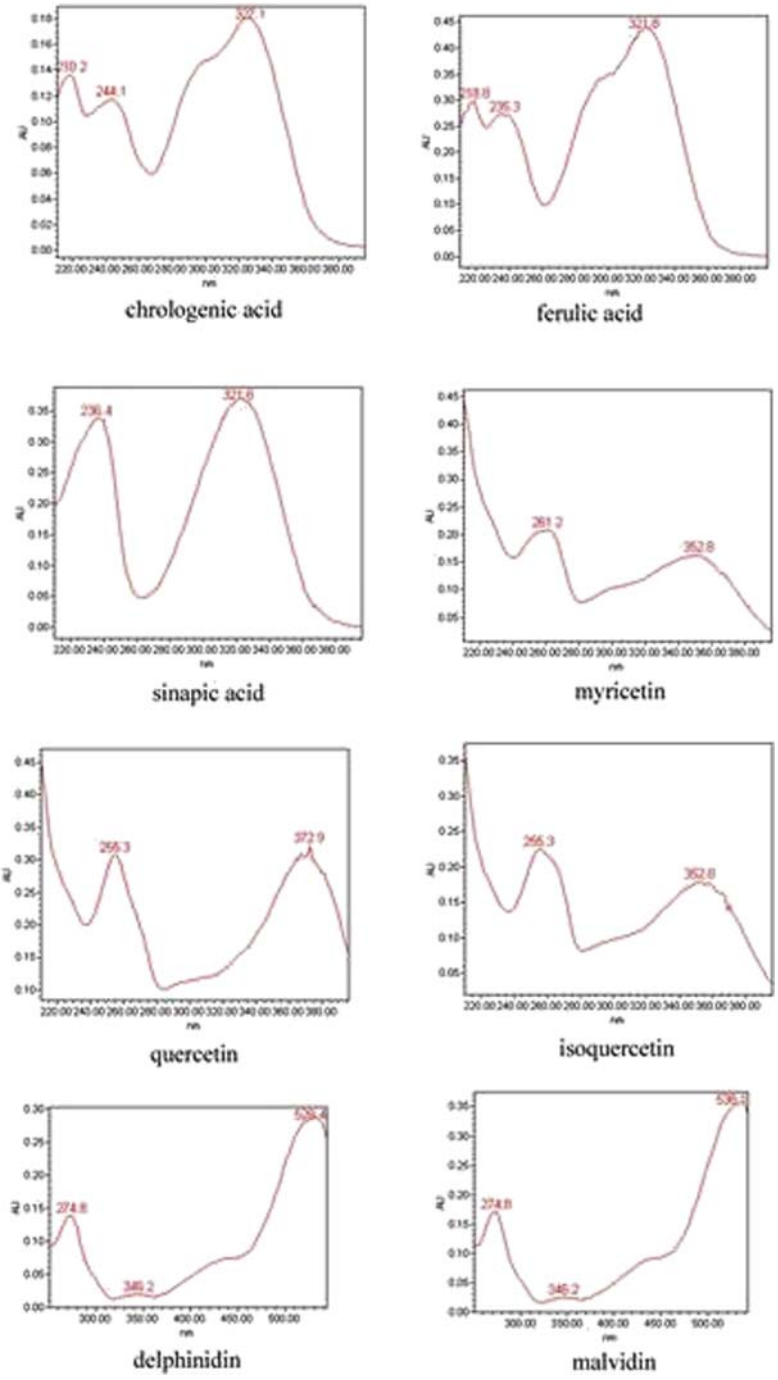

FIGURE 14.1

UV spectra of some polyphenols.

followed by malvidin (22.8%) and pelargonidin (22.1%), cyanidin (5.38%), peonidin (2.74%), and delphinidin (0.15%).

14.3.2 Fluorescence detection

Fluorescence detection is rarely used to analyze polyphenols since only a few exhibit natural fluorescence, including isoflavones without an OH group at position 5 (de Rijke *et al.*, 2002) and flavonoids with an OH group at position 3 (Sengupta and Kasha, 1979), such as catechin and methoxylated flavones (Huck and Bonn, 2001). The analysis of these compounds by HPLC-fluorimetric detection is a more selective and sensitive technique for complex mixtures and provides lower limits of detection (LODs) than UV detection. Moreover, the combination of UV detection and fluorescence makes it possible to distinguish between fluorescent and non-fluorescent co-eluting compounds, and allows a more sensitive detection of the former (Rodriguez-Delgado *et al.*, 2001).

De Quirós *et al.* (2009) proposed a new method for the analysis of flavanol, procyanidin, hydroxycinnamate, flavonol, and stilbene derivatives in white wines based on HPLC-UV-fluorimetric detection. A reverse-phase (250 × 4.0 mm, 5 µm) column was used, and the mobile phases consisted of (A) water–acetonitrile–acetic acid, 67 : 32 : 1 v/v/v, and (B) water–acetic acid, 99 : 1 v/v at 0.8 ml/min. The identification and quantification of phenolic compounds was achieved by setting the UV-Vis and fluorimetric detectors at selected wavelengths. The fluorescence detector was set at λ_{em} 360 nm and λ_{ex} 278 nm for (+)-catechin, (−)-epicatechin, procyanidin B1 and procyanidin B2, and at λ_{em} 392 nm and λ_{ex} 300 nm for trans-resveratrol. The UV–Vis detector system was set at 280 nm for flavanols, procyanidins, and trans-resveratrol, 320 nm for caftaric acid and 360 nm for flavonols. The method was fully validated, providing great repeatability with an RSD lower than 5%, limits of detection from 0.02 to 0.2 µg/ml, and recoveries of 97.3%.

Another study (Ottaviani *et al.*, 2012) used an HPLC-fluorimetric detection technique to determine epicatechin metabolites in human plasma after a dietary intervention of a dairy-based cocoa drink rich in epicatechin and procyanidins. The separation was achieved with a C18 column (150 × 4.6 mm, 3 µm) using 4% (v/v) methanol and 42 mM sodium acetate and an acetonitrile gradient with a flow rate of 0.8 ml/min. The detection of (−)-epicatechin and its related metabolites was achieved following the traces of fluorescence at 276 nm excitation and 316 nm emission and UV absorption at 280 nm. This method demonstrated that (−)-epicatechin-3′-β-D-glucuronide, (−)-epicatechin-3′-sulfate, and 3′-O-methyl-(−)-epicatechin-5/7-sulfate are the predominant (−)-epicatechin-related metabolites in humans, and confirmed the relevance of the stereochemical configuration in the context of flavanol metabolism.

Nevertheless, when working with fluorescence detection, it is necessary to take into account that emission spectra of polyphenols may show pH-dependence or might undergo solvent-dependent dual emission.

To implant fluorescence detection of phenolic compounds that do not exhibit natural fluorescence, derivatization with metal cations has been used. For example,

quercetin and kaempferol can form highly fluorescent complexes with metal cations such as Al (III) (Hollman *et al.*, 1996).

14.3.3 Electrochemical detection

Electrochemical detection is another technique that can be used to analyze polyphenols in food and biological matrices, since most flavonoids are electroactive due to the presence of phenolic groups. Electrochemical detection is a selective technique and can be very sensitive for compounds that are oxidized or reduced at low-voltage (Milbury, 2001). Most flavonoids show two values of maximum detector response: the first corresponds to the oxidation of phenolic substituents on the B-ring, while the second might be due to the other, less oxidizable, phenolic groups.

In a study by Aaby *et al.* (2004), the electrochemical behavior of 20 flavonoids and cinnamic acid derivatives was correlated with antioxidant activity measured by FRAP, DPPH, and ORAC assays. The aim of the work was to determine whether the analysis of phenolic compounds by HPLC coupled with a coulometric array detector could be used to predict antioxidant activity assessed by the three tests. It was concluded that the electrochemical response at a relatively low oxidation potential (300 mV) and the cumulative response at medium oxidation potential (400 and 500 mV) showed considerable correlation with antioxidant activities, with the highest correlations being found with FRAP and DPPH assays after short reaction periods.

Another example of polyphenol analysis by electrochemical detection is given by Peng *et al.* (2005). The aim of the study was to determine phloridzin, (−)-epicatechin, chlorogenic acid and myricetin in apple juice and cider by capillary electrophoresis with electrochemical detection. The analytes were separated in 20 min in a 75cm length capillary at a separation voltage of 18 kV in a 50 mmol/l borate buffer (pH 8.7). The limits of detection ranged from 1×10^{-7} to 5×10^{-7} g/ml for all analytes. The proposed method gave high recoveries of 95–98% and good reproducibility, with an RSD lower than 3.6%.

Capannesi *et al.* (2000) compared different techniques to evaluate the phenolic content of an extra-virgin olive oil with varying storage time and conditions. The techniques used were a disposable screen-printed sensor coupled with differential pulse voltammetry, and a tyrosinase-based biosensor operating in an organic solvent, using an amperometric oxygen probe as the transducer. Electrochemical detection revealed the degradation reaction of large molecules (such as oleuropein derivatives) into smaller ones.

Electrochemical detection has also been applied to biological samples, as described by Jin *et al.* The aim of the study was to validate and apply a method for the quantification of quercetin in human plasma after the ingestion of a commercial canned green tea (Jin *et al.*, 2004). The analysis was performed by an HPLC system coupled with electrochemical detection. A microbore octadecylsilica column (150 × 1.0 mm, 3 µm) was used and the mobile phase was methanol–water (4 : 6, v/v) containing 0.5% phosphoric acid, with a flow rate of 25 µl/min. Quercetin was oxidized at a detection potential of +0.5 V *versus* Ag/AgCl. The method proved highly selective and sensitive with a detection limit of 0.33 pg.

14.3.4 Mass spectrometry

Liquid chromatography coupled with mass spectrometry is an efficient method to detect and quantify phenolic compounds in plant extracts and biological fluids. The mass spectrometer ionizes the compounds to generate charged molecules and molecule fragments, measuring their mass-to-charge ratios (Ignat *et al.*, 2011; Marston and Hostettmann, 2009; Stalikas, 2010; Wolfender, 2009). Different sources can be used for compound ionization: fast atom bombardment (FAB), electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), atmospheric pressure photo-ionization (APPI), and matrix-assisted laser desorption ionization (MALDI). The detection of the compounds can be performed in positive or negative ion mode, the latter being more common in polyphenol analysis (Ignat *et al.*, 2011; Magiera *et al.*, 2012; Prasain *et al.*, 2004b; Schieber *et al.*, 2000; Sporns and Wang, 1998; Stobiecki, 2000). Table 14.2 shows a list of fragment ions of representative polyphenols and their metabolites in negative mode.

Different types of mass analyzers can be used in polyphenol analysis: single quadrupole (MS), triple-quadrupole (MS/MS), ion-trap mass spectrometers (MS^n), time-of-flight (TOF), quadrupole-time-of-flight (QTOF), Fourier transform mass spectrometry (FTMS), and *Orbitrap-based* hybrid mass spectrometers (LTQ-Orbitrap) (Liang *et al.*, 2012; Meda *et al.*, 2011; Mikulic-Petkovsek *et al.*, 2012; Van Der Hooft *et al.*, 2012; Xie *et al.*, 2011).

Quadrupoles consist of four parallel rods connected together, with voltages applied between one pair of rods and the other. Ions with a specific mass-to-charge ratio (*m/z*) will pass through the quadrupole when a particular voltage is applied. This enables quadrupoles to filter the ions en route to the detector. As well as single, triple quadrupole systems are also available, in which the first (Q1) and third quadrupole (Q3) work as filters while Q2 acts as the collision cell. The generic mode for screening in MS systems is the full scan, where a mass spectrum is acquired every few seconds, thus allowing the identification of the protonated or deprotonated molecule and consequently the calculation of the molecular weight of the substance. More sensitive modes of working in quadrupole systems include selected ion monitoring (SIM) in single quadrupole instruments and multiple reaction monitoring (MRM) mode in triple quadrupole instruments. In SIM experiments, the use of a fixed voltage allows the detection of a single *m/z,* whereas in MRM experiments, Q1 filters a precursor ion, and the Q2 is the collision cell, which produces a product ion by collision of the precursor ion with a neutral collision gas. The product ion is transferred into Q3 where only a specific *m/z* is allowed to pass.

Tandem mass spectrometry enables polyphenols to be detected and quantified in complex matrices through MS/MS techniques such as product ion scan, precursor ion scan, and neutral loss scan. A product ion scan mass spectrum contains the fragment ions generated by the collision of the molecular ion. A precursor ion mass spectrum is obtained by limiting the fragment ion to a single ion of interest. Parent ions (molecular ions) are scanned to determine which of them give the target fragment ion. Neutral loss mass spectra show fragment ions with a particular loss of mass, for example, glucoside polyphenols would have a mass loss of 162 μ, which corresponds to a glucoside. Working in MRM mode in combination with precursor

Table 14.2 List of Fragment Ions of some Polyphenols and Related Metabolites Obtained Working in Negative-Ion ESI Mode

Compound	MW	$[M-H]^-$	*m/z* ions
3,3/4-Hydroxyphenyl propionic acid glucuronide	342	341	165
3,3/4-Hydroxyphenyl propionic acid sulfate	246	245	165
3/4-Hydroxyphenyl acetic acid glucuronide	328	327	151
3/4-Hydroxyphenyl acetic acid sulfate	232	231	151
3-Hydroxybenzoic	138	137	93
4-Hydroxyhippuric acid	195	194	100
4-Hydroxybenzoic	138	137	93
8-Prenylnaringenin	340	339	219, 175, 119
Apigenin-*C*-hexoside-hexoside	594	593	503, 473, 383, 353
Apigenin-*C*-hexoside-pentoside	564	563	563, 503, 473, 443, 353
Caffeic acid	180	179	135, 107
Caffeic acid-*O*-hexoside 1	342	341	179, 135
Caffeic acid glucuronide	356	355	179
Caffeic acid sulfate	230	259	179
Carboxyacteyl tryptophan	290	289	203, 159, 142
Chlorogenic acid	354	353	191
Coumaric acid glucuronide	340	339	163
Coumaric acid sulfate	244	243	163
Cryptochlorogenic acid	354	353	191, 173, 135
Dihydrocaffeic acid sulfate	262	261	181
Dihydrocaffeic acid glucuronide	358	357	181
Dihydrocaffeic acid, 3,4-dihidroxyphenylpropionic acid	182	181	137
Dihydroxyphenyl acetic acid, homoprotocatechuic acid	168	167	123
Ethyl galate	198	197	169
Ferulic acid	194	193	134
Ferulic acid-*O*-hexoside	356	355	193, 178, 149
Ferulic acid glucuronide	370	369	193
Ferulic acid sulfate	274	273	193
Glutamylphenylalanine	294	293	164, 147, 103
Hydroferulic acid	196	195	136
Homovanillic acid	182	181	137
Hydroferulic acid 3,4-*O*-glucuronide	372	371	195
Hydroferulic acid 3,4-*O*-sulfate	276	275	195
Hydroxyphenyl acetic acid	152	151	107

Continued

Table 14.2 List of Fragment Ions of some Polyphenols and Related Metabolites Obtained Working in Negative-Ion ESI Mode—cont'd

Compound	MW	$[M-H]^-$	*m/z* ions
Hydroxyphenyl propionic acid	166	165	121
Isoferulic acid	194	193	134
Isorhamnetin	316	315	301
Isorhamnetin glucuronide	492	491	315
Isorhamnetin sulfate	396	395	315
Isoxanthohumol	354	353	233, 165, 119
Kaempferol	286	285	251
Kaempferol-3-*O*-rutinoside	594	593	593, 285
Kaempferol-*O*-rutinoside-hexoside	756	755	593, 285
m-Coumaric acid	164	163	119
Naringenin	272	271	151, 119
Naringenin 4′-glucuronide	448	447	271
Naringenin 7-glucuronide	448	447	271
Naringenin-7-*O*-glucoside (prunin)	434	433	433, 271
Neochlorogenic acid	354	353	191, 179, 135
p-Coumaric acid	164	163	119
Phenyl acetic acid	136	135	91
Phenyl acetic glucuronide	312	311	135
Phenyl acetic sulfate	216	215	135
Phloretin-*C*-diglycoside	598	597	477, 387, 357, 417
Piceid	390	389	227, 185
Quercetin	302	301	301, 151
Quercetin sulfate	382	381	301
Quercetin-3-*O*-glucuronide	478	477	301
Resveratrol	228	227	185, 143
Rutin	610	609	609, 300
Rutin-*O*-hexoside-pentoside	904	903	741, 609, 300
Taxifolin	304	303	285
Xanthohumol	354	353	233, 119

ion scan and product ion scan can be helpful in characterizing a particular compound found in complex mixtures. This kind of mass analyzer is the most commonly used.

An ion-trap mass spectrometer (MS^n) consists of a chamber with two electrodes and two end pieces that trap ions with a series of electromagnetic fields. Once the ions are inside, another magnetic field is applied, and only selected ions remain in the chamber. This mass analyzer is useful for structural elucidation purposes, performing multiple stage MS^n (Anari *et al.*, 2004; Wolfender, 2009).

The introduction of high-resolution spectrometers like TOF, QTOF, FTMS, and LTQ-Orbitrap has provided increased resolution and mass accuracy. A TOF mass

analyzer consists of an ion source and a detector. The ions are accelerated towards the detector with the same amount of energy through an accelerating potential. Ions with different *m/z* reach the detector at different times, with lighter ions arriving first due to their greater velocity. This spectrometer permits the analysis of a wide mass range, supplying molecular formula information and precise ion trace extraction.

The QTOF is a hybrid configuration of the TOF spectrometer. The ions are filtered in Q1, the collision takes place in Q2, and finally the product ion is determined by TOF. Compared to a triple quadrupole spectrometer, the QTOF offers greater sensitivity and accuracy when working in full scan mode, and unlike TOF equipment, measures MS/MS. The QTOF spectrometer is useful for the characterization of molecules with a wide range of mass.

FTMS is based on the effect of a magnetic field on an ion rotating in a radiofrequency field. Using the magnetic field, the ions are directed to a chamber where they rotate, describing small orbits with minimum frequency. The application of a radio frequency signal excites the ions to describe spiral orbits with increasing amplitude. When the diameter of the orbit is equal to the distance between the two electrodes, the ions are detected, generating an image of power, which is a direct function of their *m/z* relationship. This image is integrated by a Fourier transformation and converted to a signal proportional to its intensity. The full spectrum is obtained by scanning a radio frequency field that varies between 8 kHz and 100 MHz. The main advantages of this type of analyzer are its high precision mass measurements (0.001% and above) and almost unlimited resolving power.

The LTQ-Orbitrap, which combines an ion-trap analyzer with FTMS, allows MS and MS^n analysis with an error of less than 2 ppm. LTQ-Orbitrap-MS is a good tool for qualitative analysis, facilitating the structural elucidation of unknown compounds (Peterman *et al.*, 2006).

14.3.4.1 HPLC-MS

A liquid chromatograph coupled to a single quadrupole mass spectrometer allowed the identification of trans-resveratrol and cis-resveratrol (up to 10 µg/l) and trans-piceid and cis-piceid (up to 3 µg/l) in red and white wines. Separation was performed in a C18 (50 × 2.0 mm i.d., 5 µm) column, using a mobile phase of (A) water (0.5 ml/l acetic acid) and (B) acetone : acetonitrile : acetic acid (70 : 30 : 0.4 ml). Flow-rate was set at 500 µl/min.

A large number of phenolic compounds were successfully identified in propolis samples from different countries using HPLC-MS. The flavonoids were separated in a C18 column (150 × 4.6 mm, 4 µm) with (A) 0.25% acetic acid and (B) methanol as the mobile phase at a flow rate of 0.5 ml/min (Volpi and Bergonzini, 2006).

Anthocyanidins of 15 grape juice samples, four grape berries, and four grape skins were quantified using LC-MS. Separation was performed in an amide-C18 column (250 × 4.6 mm, 5 µm) with a mobile phase of (A) 0.4% TFA (v/v) in water and (B) 0.4% TFA (v/v) in acetonitrile at 1 ml/min. The results indicated that anthocyanidin concentration was higher in grape skins than the corresponding berries, and varied among the different grape juice samples (Xu *et al.*, 2012).

14.3.4.2 HPLC-MS/MS

The triple quadrupole mass spectrometer has been widely used because it provides higher selectivity, accuracy, and reproducibility, and better limits of detection and quantification, compared with a single quadrupole mass spectrometer.

A liquid chromatograph coupled to a triple quadrupole mass spectrometer equipped with a Turbo Ionspray source in negative-ion mode was used to study the levels of phenolics in different varieties of tomato (Vallverdú-Queralt *et al.*, 2011e), diced tomatoes (Vallverdú-Queralt *et al.*, 2011c), and tomato sauces (Vallverdú-Queralt A. *et al.*, 2012b). It was also used to evaluate the effects of storage on phenolic compounds (Vallverdú-Queralt *et al.*, 2011a) and the effects of pulsed electric fields on tomato polyphenols (Vallverdú-Queralt *et al.*, 2012c). Separation was performed in a C18 column (50×2.0 mm i.d., 5 μm) with a flow rate of 0.4 ml/min. Mobile phases consisted of (A) 0.1% formic acid in Milli-Q water and (B) 0.1% formic acid in acetonitrile. These conditions varied slightly depending on the product being analyzed. First, the presence of polyphenolic compounds was tested by MS/MS experiments of precursor ion scan, neutral loss scan, and product ion scan. The main objective of precursor ion scan experiments is to identify compounds belonging to a group of substances. In neutral loss experiments, the loss of 162 μ or 176 μ is used to confirm the loss of glucose, or galactose and glucuronides, respectively. Finally, product ion scan allows the identification of aglycones by comparison of their MS/MS spectra with those corresponding to the standards after typical fragmentations. MS/MS experiments were carried out by collision-activated dissociation (CAD) of selected precursor ions in the collision cell of the triple quadrupole mass spectrometer and the mass was determined by the second analyzer of the instrument. MS/MS has been used to analyze phenolic compounds in cocoa (Sanchez-Rabaneda *et al.*, 2003b), fennel (Parejo *et al.*, 2004), and artichoke (Sanchez-Rabaneda *et al.*, 2003a). Data was collected in MRM mode for quantification purposes, tracking the transition of the specific parent and product ions for each compound.

HPLC-MS/MS techniques can also be applied to determine phenolic compounds as potential taxonomical markers in food and plant samples (Andres-Lacueva C. *et al.*, 2002; De la Presa-Owens *et al.*, 1995; Romero-Perez *et al.*, 1996; Russo *et al.*, 1998; Singleton and Trousdale, 1983; Vallverdú-Queralt *et al.*, 2011e). For instance, phenolic and hydroxycinnamic acids, flavonoids, total polyphenols, and hydrophilic antioxidant capacity can be used as chemotaxonomic tomato markers to distinguish between tomato varieties (Vallverdú-Queralt *et al.*, 2011e). The polyphenolic profile determined by HPLC revealed a similarity within grape varieties and differences between varieties. Similarly, varieties of white musts (De la Presa-Owens *et al.*, 1995), wines (Romero-Perez *et al.*, 1996), and sparkling wines (Parejo *et al.*, 2004) have been shown to have different phenolic profiles.

Apart from food analysis, HPLC-MS/MS is widely used for biological fluid analysis due to its high sensitivity. In this way, an HPLC-MS/MS was used to identify and quantify phenolic compounds and metabolites from different tomato sauces in human plasma and urine in an intervention study (Tulipani *et al.*, 2012). There were three interventions in this prospective randomized, cross-over study: tomato sauce elaborated without oil, and with the addition of 5% virgin olive oil or refined olive

oil. Chromatographic separation was achieved in a C18 (50×2.0 mm, 5 µm) column, with a gradient elution of 0.1% aqueous formic acid and 0.1% formic acid in acetonitrile, and a flow-rate of 0.6 ml/min. Naringenin, ferulic acid, caffeic acid, and their corresponding glucuronide metabolites were detected in urine after the ingestion of the tomato sauces, while only two of the six urinary phenolic metabolites were identified in plasma as can be seen in Figure 14.2. Polyphenol levels of between 300 and 727 nmol/l have been detected using HPLC-MS/MS with a triple quadrupole instrument, thus showing the high sensitivity and selectivity of this system in the analysis of polyphenols in biological samples with a simple SPE extraction and clean-up process.

Using a triple quadrupole mass spectrometer, Aura *et al.* (2002) demonstrated that fecal microflora can deconjugate rutin, isoquercitrin, and quercetine glucuronides *in vitro* due to the presence of β,D-glucosidase, α,L-rhamnosidase, and β,D-glucuronidase. Fecal samples were freeze-dried before analysis. Then, polyphenol metabolites underwent liquid–liquid extraction using methanol/water (90 : 10, v/v) and were concentrated with a rotary evaporator. Samples were filtered and injected into the HPLC system. Chromatographic separation was performed using a reverse-phase column (100×1 mm). Mobile phases consisted of (A) 10 mmol/l ammonium acetate in water with 0.2% (v/v) acetic acid and (B) 10 mmol/l

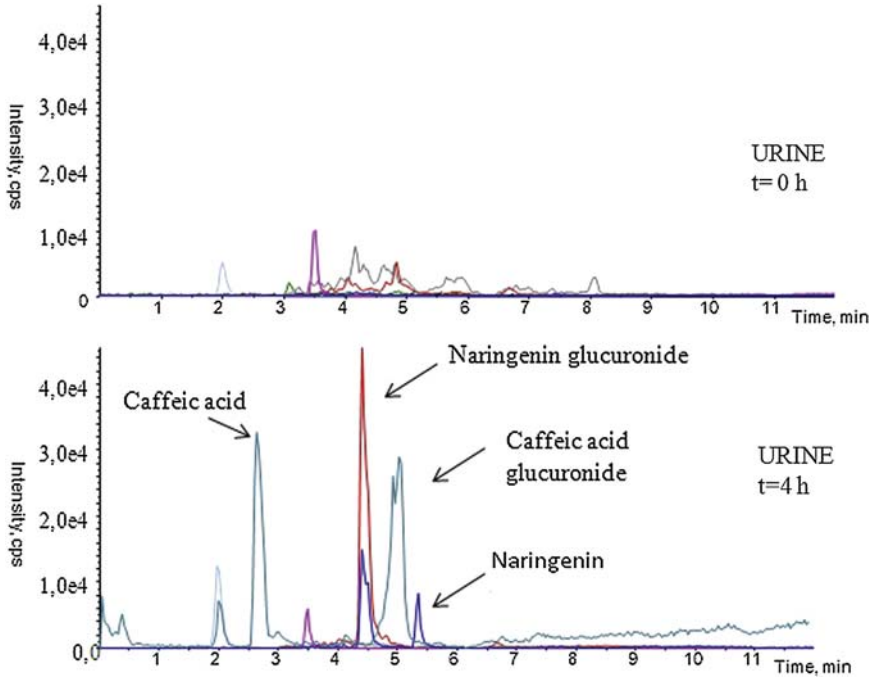

FIGURE 14.2

MRM chromatograms of urine sample at baseline (t = 0 h) and at 4 h after tomato sauce consumption.

ammonium acetate in methanol with 0.2% (v/v) acetic acid. Methanol with 0.1% (v/v) ammonium hydroxide was added as a post-column solvent (30 µl/min) to promote the desprotonation process of phenols previous to the ESI source. The measurements were performed in negative-ionization mode, and analyses were conducted in MRM. One or two fragment ions from the product ion spectra of the metabolites were used to identify the metabolites. This study showed that deconjugation and conversion of isoquercitrin and quercetin glucuronides to hydroxyphenylacetic acid occurs very rapidly in *in vitro* colonic fermentation. In contrast, rutin is deglycosylated at a slower rate, suggesting that rutin would be hydrolyzed at a slower rate than the other substrates. Therefore, the resulting quercetin aglycone appeared only transiently before further metabolism.

14.3.4.3 HPLC-HRMS

High-resolution mass spectrometry (HRMS) is used for qualitative analysis. A widely used technique is liquid chromatography/electrospray ionization–time-of-flight–mass spectrometry (HPLC-ESI-QTOF). QTOF technologies allow exact mass measurements of both MS and MS/MS ions. QTOF-MS has been used to determine phenolic compounds in gazpachos, ketchups, and tomato juices (Vallverdú-Queralt A. *et al.*, 2011b), resulting in the identification for the first time of apigenin-*C*-hexoside-hexoside and apigenin-*C*-hexoside-pentoside in tomato-based products. These compounds were distinguished by the presence of the ion $[M-H-60]^-$ (Figure 14.3A and B), following the method of Han *et al.* (2008) which involves liquid chromatography coupled with electrospray ionization mass spectrometry. Vallverdú-Queralt *et al.* (2011b) reported for the first time the presence of protocatechuic acid-*O*-hexoside,

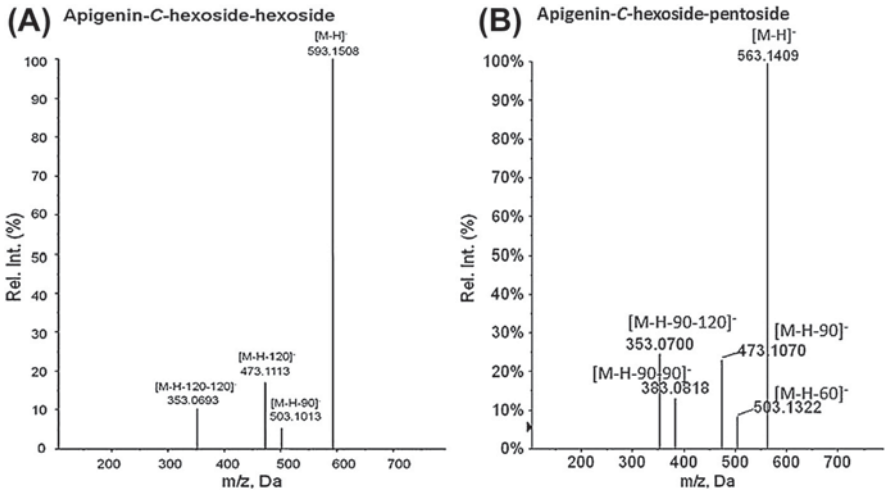

FIGURE 14.3

(A) Mass spectrum of apigenin-*C*-hexoside-hexoside. (B) Mass spectrum of apigenin-*C*-hexoside-pentoside.

caffeic acid-*O*-dihexoside, apigenin-*C*-hexoside-hexoside and apigenin-*C*-hexoside-pentoside in tomato-based products.

Metabolomics, a combination of analytical and statistical techniques, facilitates sample differentiation by quantitatively and qualitatively measuring the dynamic range of metabolites. With the recent developments in plant metabolomic techniques, it is possible to detect several metabolites simultaneously and reliably compare samples for differences and similarities in a semi-automated and untargeted manner. Metabolomics is predicted to play a crucial role in "bridging the phenotype–genotype gap" and in achieving complete genome sequence annotation and the understanding of gene function (Hall, 2006).

Metabolomics has also been used in the quality control of medicinal plants (Kim *et al.*, 2011). An ultraperformance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-QTOF MS)-based metabolomic technique was applied for the metabolite profiling of 60 *Panax ginseng* samples of 1 to 6year-old plants. After submitting the data for classification by various metabolite selection methods, the results showed variations according to the age of the samples, especially for those of 4 to 6year-old plants. Thus, a UPLC-QTOF MS-based metabolomics approach was able to quickly and accurately distinguish between *P. ginseng* samples according to their cultivation period.

HPLC-ESI-QTOF has also been used as a non-targeted strategy to differentiate between organic and conventional ketchups (Vallverdú-Queralt *et al.*, 2011d). Interpretation of the observed MS/MS spectra in comparison with results found in the literature was the main tool for putative identification of metabolites. The compounds found in significantly higher ($p<0.05$) amounts in organic than in conventional ketchups were: caffeoylquinic and dicaffeoylquinic acids, caffeic and caffeic acid hexosides, kaempferol-3-*O*-rutinoside, ferulic-*O*-hexoside, naringenin-7-*O*-glucoside, naringenin, rutin, and quercetin. Examination of the chromatograms in TOF-MS mode also suggested the presence of glutamyl phenylalanine (*m/z* 293) and *N*-malonyltryptophan (*m/z* 289).

Recently, LTQ-Orbitrap-MS has been proposed as one of the most suitable strategies for qualitative analysis, since it routinely delivers the highest resolution and mass accuracy, which are necessary to reduce analysis times and increase confidence in results. Due to its ability to eliminate interference in the initial mass selection stage and to the specificity of MS/MS measurements, this spectrometer facilitates qualitative analysis of nontarget compounds. Elemental composition assignment and exact mass measurements are essential for molecule characterization. The structural elucidation of unknown compounds is easily accomplished by using accurate mass measurement of the product ions formed in MS^n experiments.

Thus, accurate mass experiments have yielded the elemental composition of polyphenol compounds, with MS^n fragment ions providing additional structural confirmation. The LTQ Orbitrap provides accurate mass MS and MS^n spectral data on a chromatographic time-scale with scan cycles of 1 s (at R = 60,000) or less. Up to five to eight sequential fragmentation spectra can be obtained,

depending on the concentration and ionization efficiency of the compound. Using multiple-stage mass spectra it is possible to generate spectral trees of the compounds (Sheldon *et al.*, 2009). Van der Hooft *et al.* (2011) validated and applied an accurate mass MS^n spectral tree approach to 121 polyphenolic compounds of different chemical flavonoid subclasses, including isomeric forms. The study focused on the possibility of discriminating between positional and stereoisomeric forms. Accurate mass spectra of polyphenols were obtained using an LTQ-Orbitrap hybrid mass spectrometer in negative and positive ionization mode. The accurate MS^n fragmentation spectra enabled isomeric compounds to be differentiated. Spectral trees of 119 polyphenols (except catechin and epicatechin) showed unique fragments and differences in relative intensities of fragment ions. Thus, spectral trees constitute a potent tool for the identification of phenolic compounds or their metabolites. This tool could be applied to generate an MS^n metabolite database based on MS^n fragmentation and exact mass measurement.

Another study used liquid chromatography coupled with an LTQ Orbitrap to analyze polyphenols in tomato samples (Vallverdú-Queralt *et al.*, 2010). A C18 column (50 × 2.0 mm i.d, 5 µm) was used to separate the compounds. Gradient elution was performed with water/0.1% formic acid and acetonitrile/0.1% formic acid at a constant flow rate of 0.4 ml/min. A total of 38 compounds were identified in the tomato samples with very good mass accuracy (< 2 mDa). The spectra generated for cinnamic and benzoic acids showed the deprotonated molecule $[M\text{-}H]^-$ and some fragments. The typical loss of CO_2 was observed for gallic, protocatechuic, caffeic, and ferulic acids, giving $[M\text{-}H\text{-}44]^-$ as a characteristic ion, and loss of a methyl group $[M\text{-}H\text{-}15]^-$ was observed for ferulic acid. Flavonol aglycones such as quercetin gave the deprotonated molecule $[M\text{-}H]^-$ as a characteristic ion and ions corresponding to retro-Diels Alder fragmentation in the C-ring involving 1,3 scission, as described by other authors (Gruz *et al.*, 2008). The LTQ-Orbitrap was crucial for the structural determination of kaempferol-*O*-rutinoside-hexoside and rutin-*O*-hexoside-pentoside, which were not discernible under lower-resolution conditions.

Phloridzin-*C*-diglycoside (*m/z* 759) was only identified in the LTQ-Orbitrap due to the lack of sensitivity of the triple quadrupole. Figure 14.4 shows the MS^2 of *m/z* 759 of phloridzin-*C*-diglycoside, displaying losses of 90 u and 120 u from *m/z* 759 and 639, respectively, which confirmed the presence of two hexose units. Moreover, loss of H_2O was observed in the product ion spectra of *m/z* 759, showing an ion at *m/z* 741, which displayed a loss of 120 u (*m/z* 621). Losses of 90 and 120 u are characteristic fragment ions in the MS/MS mode of *C*-glycosides (Parejo *et al.*, 2004; Sanchez-Rabaneda *et al.*, 2003a).

In another study, 53 *O*-glycosyl-*C*-glycosyl flavones with *O*-glycosylation on phenolic hydroxyl or on the *C*-glycosyl residue, or a combination of both forms, were studied by liquid chromatography-UV diode array detection-electrospray ionization mass spectrometry ion trap in the negative mode. The study of the relative abundance of the main ions from the MS preferential fragmentation on -MS^2 and/or -MS^3 events allowed the differentiation of the *O*-glycosylation position,

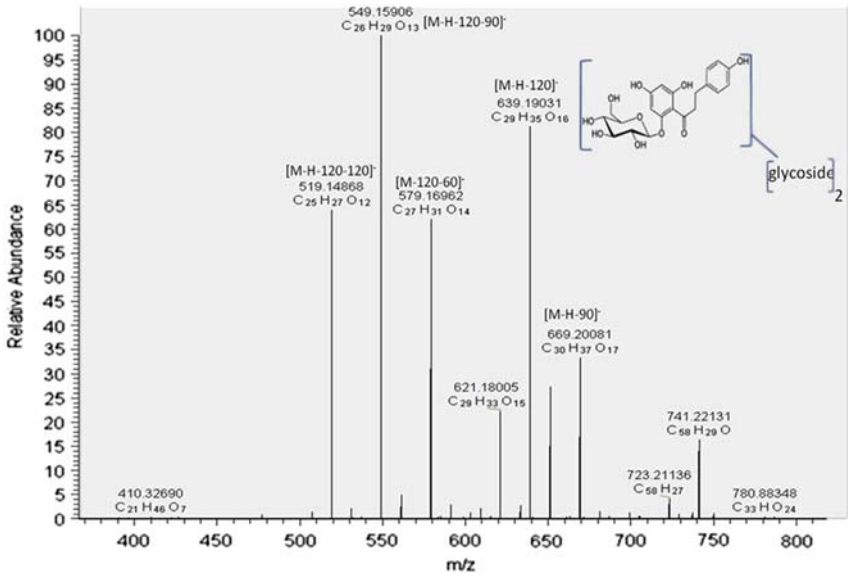

FIGURE 14.4

Identification of phloridzin-*C*-diglycoside in tomato samples. The MS^2 in product ion scan of *m/z* 759 shows the characteristic fragment ions of a *C*-diglycoside polyphenol.

either on a phenolic hydroxyl or on the sugar moiety of *C*-glycosylation (Ferreres *et al.*, 2007).

De Paepe *et al.* (2013) developed and validated a method to identify phenolic compounds in apple extracts using UHPLC coupled with an Orbitrap. An accurate mass spectrometry technique allowed the identification of 39 phenolic compounds in apples, including flavonoids, proanthocyanidins, and phenolic acids.

14.4 Ultra-high-performance liquid chromatography (UHPLC)

Liquid chromatographic performance has been improved by the introduction of UHPLC. To improve chromatographic separation, new columns with a very small particle packing size (1.7 μm) have been developed. Column efficiency is inversely proportional to particle size as the Van Deemter equations prove, so columns with 1.7μm particles provide higher resolution and better efficiency than the conventional ones (Novakova *et al.*, 2006). Improved mobile phase systems can operate at high backpressures (15000 psi), thus enhancing mobile phase viscosity and the capacity to dissolve analytes (Epriliati *et al.*, 2010). Due to these high pressures, new hardware in LC technology has been developed. As a result of the combination of columns and high pressure, UHPLC has enhanced sensitivity and peak resolution, and reduced

both analysis time and costs (Gruz *et al.*, 2008; Leandro *et al.*, 2006; Ortega *et al.*, 2010; Wu *et al.*, 2008).

14.4.1 UHPLC-UV

An ultra high pressure liquid chromatography technique was used to develop a new method for analyzing conjugated isoflavones in commercial soy milks (Toro-Funes *et al.*, 2012) using UV detection. This approach allowed the determination of 12 isoflavones in less than 8 min in a single run. The method was fully validated, with limits of detection lower than 0.05 mg/l and a limit of quantification below 0.2 mg/l. Chromatographic separation of analytes was achieved using a C18 column (50 × 2.1 mm, 1.7 µm). Solvent A was ultrapure water with 0.1% formic acid, and solvent B was acetonitrile with 0.1% formic acid, and the flow rate was set at 0.6 ml/min. The quantification of isoflavones was performed at 262 nm.

Gonçalves *et al.* (2013) also developed a new method to analyze hydroxybenzoics and hydroxycinnamics acids in wine samples using UHPLC coupled to a photodiode array detector in 11 min. The method was validated and showed limits of detection of 0.01–0.2 mg/l and a limit of quantification of 0.03–0.7 mg/l. A C18 (100 × 2.1 mm, 1.8 µm) column was used, and the gradient elution was performed with 0.1% formic acid and methanol as mobile phases at 0.25 ml/min. Before UHPLC analysis, phenolic compounds were extracted using microextraction by packed sorbent optimized for hydroxybenzoic and hydroxycinnamic acids.

Another study used UHPLC coupled with a UV detector to identify and quantify 58 polypyhenols in sage tea (Zimmermann *et al.*, 2011) within 28 min. Separation of the phenolic compounds was carried out using a reverse-phase column (150 × 2.1 mm, 1.7 µm). 0.1% formic acid was used as the aqueous mobile phase, and acetonitrile containing 0.1% formic acid as the organic mobile phase, at a flow rate of 0.4 ml/min. Wavelengths of the UV detector were set for quantification at 273, 320, and 360 nm. The method was applied to characterize 16 commercial brands of sage tea.

14.4.2 UHPLC-MS/MS

Coupling UHPLC with electrospray ionization tandem mass spectrometry offers a strong alternative to conventional HPLC-MS/MS in terms of analysis time, costs, and improved resolution and sensitivity.

Using UHPLC-MS/MS, 17 phenolic acids were quantified in white wine, grapefruit juice and green tea infusion within 10 min. Separation was performed in a reverse-phase column C8 (2.1 × 150 mm, 1.7 µm) with a mobile phase of aqueous 7.5 mM HCOOH (A) and acetonitrile (B) at a flow rate of 250 µl/min (Gruz *et al.*, 2008). The validated method offered good precision and accuracy, and limits of

detection of 0.15–15 pmol/injection. A UHPLC-MS/MS system is also suitable for routine analysis in laboratories.

A UHPLC-MS/MS was used to analyze procyandins and alkaloids (caffeine and theobromine) in samples of cocoa nibs (Ortega *et al.*, 2010). A high-strength silica separation column (100 × 2.1 mm i.d., 1.8-mm particle size) was used with (A) water/acetic acid (99.8/0.2, v/v) and (B) acetonitrile at a flow rate of 0.4 ml/min. This method allowed catechin and epicatechin to be quantified separately, which was not possible with HPLC-UV due to the coelution of the compounds. Also, the total analysis time for a cocoa phenolic extract was reduced from 80 min by HPLC to 12.50 min by UHPLC, and limits of detection of the procyanidin were enhanced from 0.009–0.02 mg/ml with HPLC-MS/MS to 0.007–0.01 mg/ml with UHPLC-MS/MS.

Twenty-six phenolic compounds, including 15 isoflavonoids, five flavones, four flavanones, a coumestan, and a coumarine of plant material, were analyzed in 17 min by UHPLC–ESI–MS/MS. The 26 compounds were separated with a C8 column (2.1 × 150 mm, 1.7 mm), using a mobile phase of methanol (A) and 10 mM aqueous formic acid (B) 100% A at a flow rate of 0.2 ml/min. The validated method achieved good accuracy and precision, with limits of detection ranging between 0.0001 and 10 pmol/injection. Solvent consumption and time were reduced compared to conventional HPLC systems (approximately 30–70 min) (Prokudina *et al.*, 2012).

14.5 Conclusions

Polyphenol characterization is a difficult task due to the large number of phenolic compounds present in nature and the few available commercial standards.

Efficient identification and quantification involves pretreating the samples to avoid interference. Food and beverage samples are normally analyzed after liquid–liquid or solid–liquid extraction, but biological samples such as plasma, urine, or serum usually require solid-phase extraction, since they contain far lower levels of compounds than food samples. Solid-phase extraction procedures are also used to clean up the samples by eliminating interfering compounds, thus avoiding the matrix effect.

Polyphenols can be identified or quantified with various analytical techniques, but the most commonly used are liquid chromatography coupled with UV detection, and liquid chromatography coupled with mass spectrometry.

The most widespread technique for analyzing polyphenols in food and plant samples is HPLC-UV, which also constitutes an excellent option for routine analysis in analytical laboratories and the food industry. In contrast, when working with biological samples, a more sensitive technique like mass spectrometry is required due to the complexity of the matrix and because mass spectrometry offers lower limits of detection than HPLC-UV (Table 14.1).

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3.1.3. Polyphenols, mortality and cardiovascular diseases. Latest evidences

Polyphenols and major health outcomes

Polyphenols are not essential for short-term health status, unlike vitamins and other micronutrients, however there is increasing evidence that long-term intake may reduce the incidence of chronic diseases such as cancer, type-II diabetes, cardiovascular diseases (CVD) and neurodegenerative diseases[4,5].

Bioavailability, absorption and metabolism of polyphenols

The bioavailability and metabolism of dietary polyphenols must be considered when exploring their possible health benefits. It has been estimated different bioavailability rates according to the class of polyphenols. Thus, isoflavones have the highest bioavailability (33-100%), followed by flavonols (12-41%), flavanones (11-16%) and monomeric flavanols (2-8%)[14].

It has been estimated that 90-95% of dietary polyphenols are absorbed in the colon and not in the small intestine[15]. Most of dietary polyphenols are glycosylated in the food matrices and their absorption is associated with cleavage and release of the aglycone. Before passage into the blood stream, the aglycones undergo some degree of phase II metabolism in the enterocytes forming sulfate, glucuronide, and/or methylated metabolites. There is also efflux of some of the metabolites back into the lumen of the small intestine[16]. Polyphenols that are not absorbed in the small intestine are transported to the colon where are also exposed to microbial degradation processes such as hydrolysis, microbial deconjugation and microbial conversion. Once in the portal vein, metabolites reach the liver, where they can be subjected to further phase II metabolism, and enterohepatic recirculation may result by recycling back to the small intestine through bile excretion[17] (**Figure 2**).

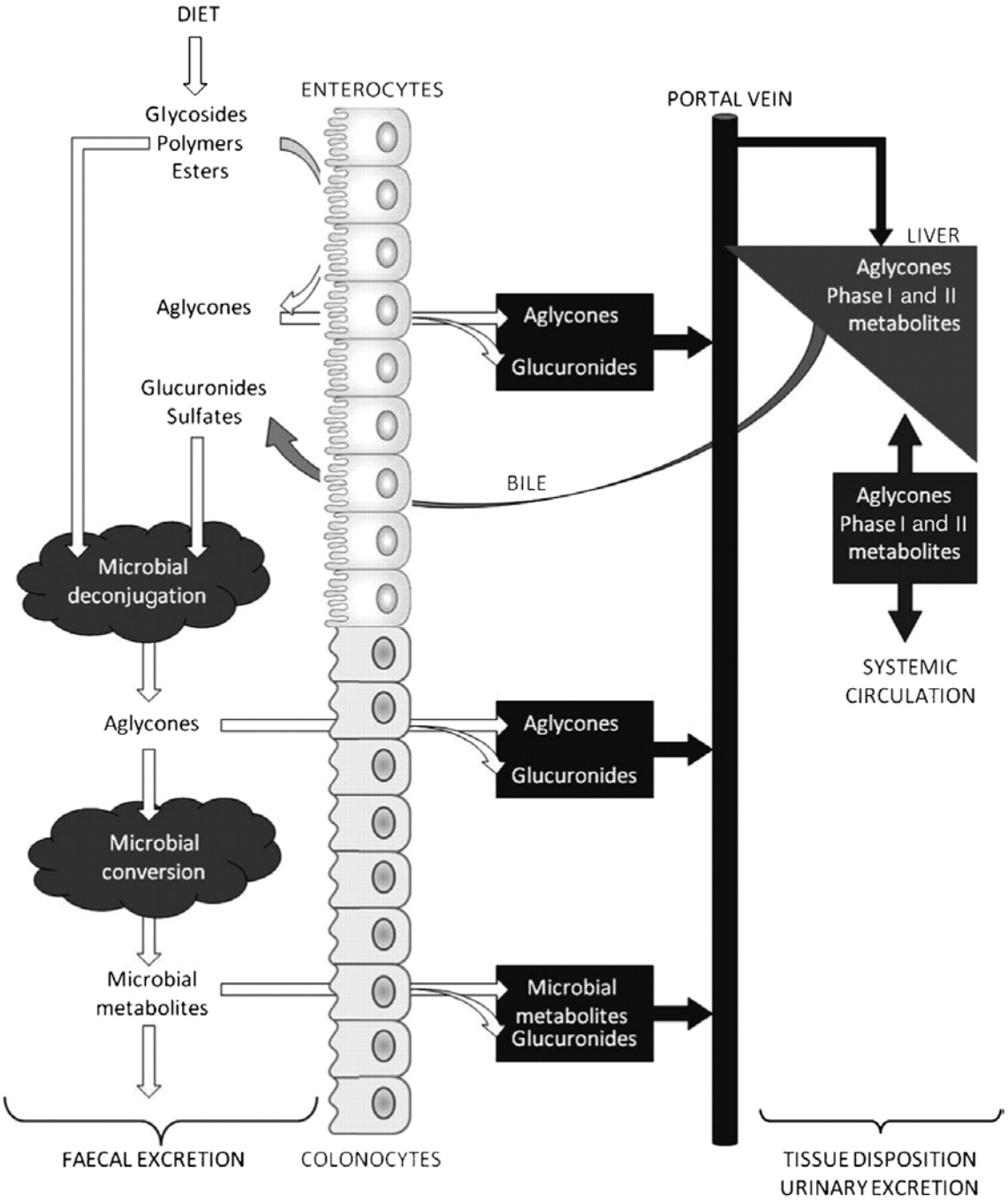

Figure 2: Metabolic fate of polyphenols. Source: Kemperman R.A *et al*. 2010[18]

Polyphenols and Cardiovascular diseases

According to the World Health Organization[1], CVD are the principal cause of death in the world. In 2012, 17.5 millions of people died for CVD, which represents a 31% among all registered deaths. Of these deaths, 7.4 million were due to coronary heart disease, and 6.7 million to strokes. The incidence of CVD has not decreased significantly in recent years, thus it is necessary to persist in promoting primary prevention. The major causes of CVD are age, sex, smoking, hypertension, overweight and obesity, type-II diabetes, dyslipidemia (high levels of LDL-

cholesterol and/or low levels of HDL-cholesterol), family history of CVD, sedentary lifestyle and diet. Although some of these risk factors are not modifiable, most of the risk factors depend on the lifestyle and habits and they are strongly related to each other. Therefore, a calm life, sleep well, exercise regularly, follow a balanced diet and not to smoke contribute in reducing hypertension, dyslipidemia or diabetes, which all of them are CVD risk factors[19].

Polyphenols were first considered as simple antioxidants since they clearly demonstrated reactivity with radicals and metal-chelating properties in *in vitro* assays. However, nowadays, they and their related metabolites are recognized as signaling molecules[14,20] since the following considerations contradict the direct antioxidative effect *in vivo*:

- Low concentration of polyphenols in the systemic circulation and body tissues.
- High level of metabolism or biotransformation that polyphenols undergo during absorption and distribution, which blocks catechol like moieties and the ability of delocalize unpaired electrons.
- Lack of evidence regarding the efficacy in long-term clinical trials compared to other well-known antioxidants such as vitamin C, vitamin A and vitamin E.

Therefore, growing evidence suggest that polyphenols may have alternative mechanisms of action in CVD risk reduction including direct anti-inflammatory effects, modulation of intracellular signaling pathways and genetic expression, nitric oxide (NO) homeostasis, and platelet antiaggregant capacity (**Figure 3**)[21,22].

Vasodilatory effect of polyphenols

Endothelial dysfunction plays a key role in the development of hypertension, which is one of the most important risk factors in CVD. Endothelial dysfunction is defined as an imbalanced between endothelium-derived contracting factors like endothelin (ET-1), and relaxing factors such as NO. NO is produced by endothelial nitric oxide synthase (eNOS) from L-arginine and induces blood vessels dilation through cyclic guanylyl monophosphate (cGMP) pathway, leading to a decrease in blood pressure[23].

New evidences revealed that polyphenols cause NO-mediated endothelium-dependent relaxations and increase endothelial formation of NO. Wine, grape juice and grape skin extracts induce relaxation in rat aortic rings with endothelium in a concentration-dependent manner[24]. The grape-derived products increased the synthesis of endothelial NO, which relaxed the vascular smooth muscle via cGMP-mediated pathway. These endothelium-dependent relaxations are correlated with the polyphenol concentrations in red wine and have been further observed in various types of animal blood vessels[25–27]. Moreover, other food products

rich in polyphenols, such as cocoa, tea, and chokeberry, have also showed to induce endothelium-dependent NO-mediated relaxations in arteries.

Another pathway that polyphenols may decrease blood pressure is by increasing the intracellular free calcium concentration which activates endothelial NO synthase (eNOS)[28].

Anti-inflammatory effect of polyphenols

The expression of inflammatory mediators is activated under different circumstances, including oxidative stress, mitochondrial dysfunction and ischemia reperfusion, however all these mechanisms lead to the activation of the transcription factor NF-κβ (nuclear factor kappa beta). NF-κβ induces the activation of the inflammatory cascade, including: I) release of inflammatory mediators like tumor necrosis factor alpha (TNFα) and interleukins, II) expression of adhesion molecules like vascular cell adhesion molecule 1 (VCAM-1) and intracellular adhesion molecule 1 (ICAM-1), and III) activate prostaglandins pathway associated to cyclooxygenase-2 (COX-2) activity.

Polyphenols could inhibit inflammation through NF-κβ inhibition[22,29,30]. For example, procyanidins may modulate the anti-inflammatory response by molecular mechanisms including the modulation of the arachidonic acid pathway by inhibition of COX and LOX (lysyl oxidase) enzymes, inhibition of gene transcription, protein expression and enzymatic activity of eicosanoid-generating enzymes, inhibition of the production and secretion of inflammatory mediators (cytokines and NO), inhibition of mitogen-activated protein kinase (MAPK) pathway activation and modulating the expression of the nuclear factor NF-κβ[31]. Protocatechuic acid has been proposed as an antiatherogenic due to its vascular anti-inflammatory activity. Protocatechuic acid revealed to inhibit monocyte adhesion to TNFα in endothelial cells, and to reduce VCAM-1 and ICAM-1 expression and NF-κβ binding activity[32]. Furthermore, quercetin metabolites have also shown anti-inflammatory activity. 3'-*O*-methyl-quercetin and 4'-*O*-methyl-quercetin inhibited ICAM-1 expression at physiological concentrations in human aortic endothelial cells. In contrast, other quercetin metabolites like quercetin-3'-*O*-sulfate and quercetin-3-*O*-glucuronide did not inhibit adhesion molecule expression[33]. However, in another study, this metabolites showed to be able to inhibit VCAM-1 cell surface expression[34].

Antithrombotic effect of polyphenols

Platelets play an important role in the development of atherosclerosis and are often found in arterial lesion sites. They are associated with reactive oxygen species cell producers like neutrophils, monocytes, macrophages and endothelial cells. Platelet adhesion, activation and

aggregation represent the first stage of arterial thrombus formation. Adherent platelets are activated by several mediators, including collagen, adenosine diphosphate (ADP), thromboxane A_2 (TxA_2) and thrombin. Once platelets are activated, they secrete chemotaxins, clotting factors and vasoconstrictors which promote thrombin generation and more platelet accumulation. Activated platelet also change shape which help to promote further aggregation and coagulation and activates integrin complex glycoprotein IIb/IIIa (GPIIb/IIIa).

Current evidences suggest that polyphenols could inhibit platelet activation and related signal transduction pathways, neutralize free radicals, enhance oxide nitric production and block thromboxane receptors like TxA_2[22,35]. In an *ex vivo* study, catechin and epicatechin showed to reduce platelet activity by inhibiting ADP-induced expression of GPIIb-IIIa surface glycoproteins[36]. Recently, *Osterag et al.* 2013 showed that flavan-3-ol reduced ADP-induced platelet aggregation and P-selectin expression in men and decreased thrombin receptor-activating peptide-induced platelet aggregation in women[37].

Therefore, polyphenols may down-regulate thromboxane A_2 receptors reducing platelet aggregation, block ADP and GPIIb-IIIa receptors avoiding platelet aggregation, block collagen receptors preventing collagen-induced platelet aggregation and lower the expression of P-selectin[35].

Antiatherogenic effect of polyphenols

Atherosclerosis is a multifactorial chronic pathology of the blood vessel wall characterized by oxidative stress, inflammation, and immune infiltration, which is caused by LDL-cholesterol accumulation in the arterial wall. The development of atherosclerosis leads to recruitment of monocytes and gradual oxidation of accumulated LDL particles. The monocyte recruitment is promoted by the expression of adhesion molecules like VCAM-1. After adhering to the endothelium, monocytes begin to absorb accumulated to LDL. This process induces monocytes to turn into macrophages and these cells are able to release inflammatory cytokines and chemoattractants. Activated macrophages are also an important source of TxA_2, which induces proliferation of vascular smooth cell and proinflammatory signaling in endothelium, generating a vicious circle with positive feedback.

Several studies have pointed out different mechanism of action of which polyphenols may act as an antiatherogenic factor[5,38–41]. Resveratrol showed to inhibit vascular smooth cell proliferation through eNOS pathway associated to SIRT-1 activation[42]. SIRT-1 is a metabolic mediator, which induces eNOS activity and downregulates inflammatory molecules by inhibiting NF-κβ. Another study showed that polyphenols from propolis could inhibit atherosclerotic

process by improving the lipid profile and down-regulating various atherogenic mediators such as Interferon-gamma (IFN-γ), interleukine-6 (IL-6), vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDFG)[43].

In brief, all these mechanism of action of polyphenols against CVD development shared the eNOS activity modulation, inhibition of NF-κβ and down-regulation of COX-mediated generation of prostaglandins and thromboxanes, thus all of them represent therapeutic targets of polyphenols.

Polyphenols and mortality

Polyphenols intake have been associated with lower incidence of chronic diseases in several studies such as CVD, type-II diabetes and cancer. Thus, a higher consumption of polyphenols is expected to lower the risk of all-cause and expand life expectancy, however, little number of published works have study this association. Tresserra-Rimbau *et al*.[44] found an inverse association between higher consumption of total polyphenols and all-cause mortality with a mean risk reduction of 37% in an elderly Spanish population. In this study, the analysis was further stratified according to the phenol subclasses and stilbenes and lignans were the only classes that showed a significant association. Kuriyama *et al*.[45] also reported an inverse association between green tea consumption, a polyphenol rich beverage, and all-cause mortality in a Japanese population. They found that consumption of five or more cups of green tea/day reduced all-cause mortality risk by 15%. In both studies, the association was greater in women than in men. In the work of Mink *et al*.[46] performed in 34,489 postmenopausal women (Iowa Women's Health Study), anthocyanidins, flavanones, flavones, flavonols, isoflavones and flavanols significantly reduced all-cause mortality risk. Isoflavones had the greater association reducing the risk by 70%.

Several works have studied the association between intake of polyphenols or a specific class of polyphenols and cardiovascular mortality. Mursu *et al*.[47] found an inverse association between flavanone and flavone intakes, but not total flavonoid intake, and cardiovascular mortality. In the Zutphen Elderly Study, Hertog *et al.*[48] found that those in the highest consumption group of flavonoids were 68% less likely to have coronary heart disease (CHD) death compared to those in the lowest consumption group. In the Iowa Women's Health Study, they also found that intake of flavanones and anthocyanidins, but not total flavonoids, were associated with a decreased risk of CHD and CVD mortality[46]. However, a few studies did not report an inverse association between polyphenols and cardiovascular mortality. For example, in the Health Professionals

Follow-up Study, Rimm *et al*[49] did not find flavonoid intake (flavonols and flavones) to be associated with the risk of CHD mortality among male participants. This finding was further confirmed in women participants[50].

In a meta-analysis of prospective cohort studies publish in 2003 by Huxley *et al.*[51] showed that high dietary intakes of flavonols might be associated with a decreased risk of CHD mortality, reducing the risk up to 20%.

In conclusion, polyphenols or some polyphenols subclasses intake, such as anthocyanindins, isoflavones, lignans and flavonols, might reduce cardiovascular and all-cause mortality, but more studies are needed to support this hypothesis.

3.2. Beer

3.2.1. Beer consumption in Spain and Europe

Beer is a fermented beverage most commonly consumed worldwide. It is known that beer has been consumed since the Ancient Egypt and Mesopotamia era, and its consumption was firstly extended to the Mediterranean countries, then to the Northern Europe and finally to the rest of the world.

According to a report of the World Health Organization in 2014[52], in Spain it is consumed 16.4 L of alcohol per capita and year, 49.7% as beer, 20.1% as wine, 28.2% as spirits and 1.8% as other (**Figure 4**).

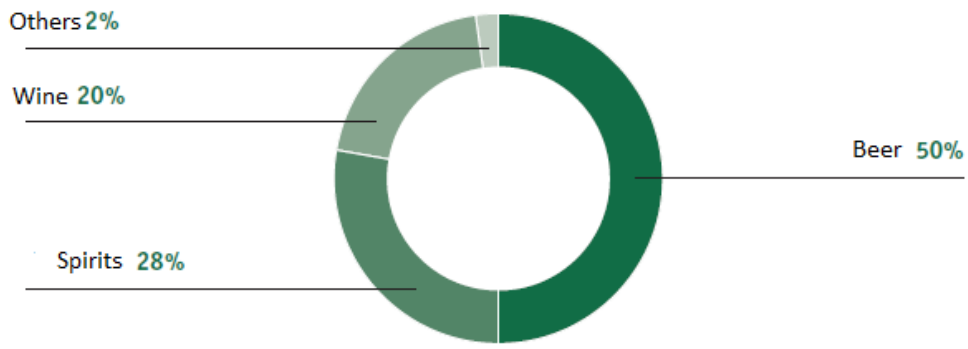

Figure 4. Alcohol consumption in Spain. Source: WHO, 2014[52]

According to the Brewers of Europe report 2016, the EU is the second largest beer producer in the world, after China. There are over 6,500 active breweries, which produced around 383 million hectoliters of beer in 2014. The brewing sector in Europe is also a major exporter. EU countries sold over 27 million hectoliters of beer outside the EU in 2014. Some countries' exports (intra and extra-EU) represent more than half of their total production (notably Belgium, the

Netherlands and Denmark). The total contribution to value added in the EU in 2014 was around €51 billion. This would be comparable for instance to the total GDP of Croatia or Luxembourg. Beer is mostly produced in Germany, United Kingdom, Poland and Spain. However, it is more consumed per capita in Czech Republic, Germany, Austria and Poland. Spain is in the 26th position of beer consumption per capita ranking (**Figure 5**).

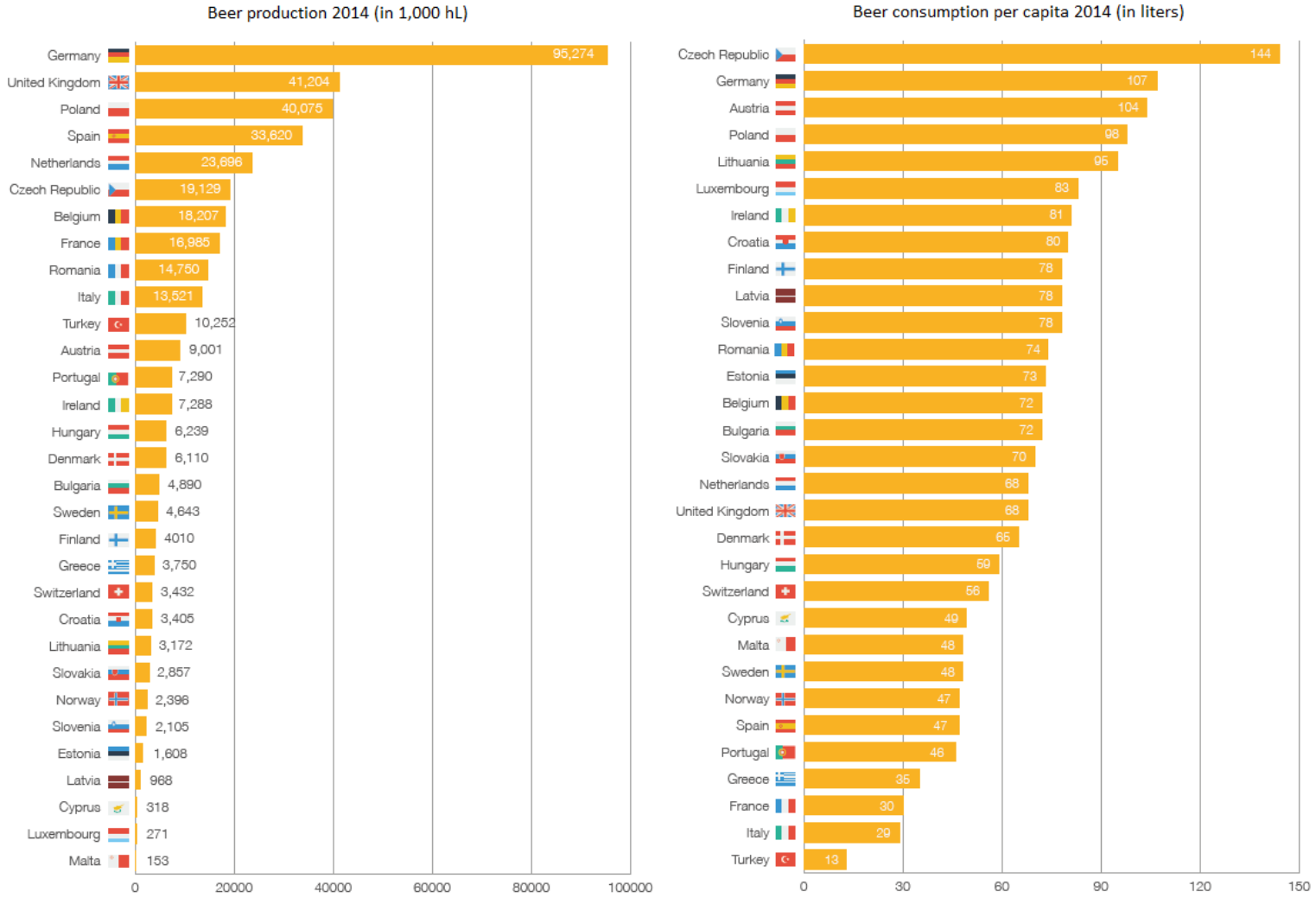

Figure 5. Beer production and consumption in Europe per country, 2014. Source: the Brewers of Europe statistics report 2015[53]

3.2.2. Beer composition

Beer is a complex mix of nutrients and bioactive compounds such as carbohydrates, amino acids, minerals, vitamins and polyphenols among others. **Table 1** shows the nutritional composition of beer, including macronutrients and micronutrients. In the following section, beer polyphenols are described in detail.

Table 1: Beer composition. Source: BEDCA Base de Datos Española de Composición de Alimentos (Spanish Food Composition Database)[54]

Nutrient (Value per 100 g)	Beer	Non-alcoholic beer
Energy	42 kcal	26 kcal
Protein	0.5 g	0.3 g
Total lipid (fat)	0 g	0 g
Carbohydrate, by difference	3.12 g	5.4 g
Fiber, total dietary	0 g	0 g
Minerals		
Calcium, Ca	8 mg	5 mg
Iron, Fe	0.01 mg	0.02 mg
Magnesium, Mg	9.6 mg	7.7 mg
Phosphorus, P	55 mg	20 mg
Potassium, K	37 mg	40 mg
Sodium, Na	4.4 mg	2.6 mg
Zinc, Zn	0.006 mg	0.008 mg
Vitamins		
Thiamin	0.003 mg	0.006 mg
Riboflavin	0.03 mg	0.005 mg
Niacin	0.43 mg	0.7 mg
Vitamin B6	0.06 mg	0.03 mg
Folic acid	6.3 µg	15 µg
Vitamin B12	0.1 µg	0.1 µg

3.2.3. Beer polyphenols

Frequently it is wrongly assumed that red wine is the only fermented beverage that contains polyphenols. Even though the concentration of polyphenols in red wine is very high, beer is also a source of dietary polyphenols and the phenolic concentration in beer is slightly higher than in white wine. According to the phenol-explorer data base[55], wine has a mean phenolic concentration of 215.5 mg/100 mL, beer has 27.8 mg/100 mL and white wine has 23.1 mg/100 mL (using Folin-Ciocalteau method).

Up to now, more than 50 phenolic compounds have been identified in beer and it has been estimated that 70-80% of the beer polyphenols come from malt and the rest 20-30% from hops[6]. The major phenolic compounds in beer are hydroxybenzoics acids and hydroxycinnamic acids such as ferulic acid, *o*-coumaric acid, 2- and 4-hydroxybenzoic acid and gallic acid 3-*O*-gallate,

and tyrosol[55] (**Table 2**). However, the most characteristic phenols of beer are hops polyphenols, such as prenylflavanoids, which concentration depends on the beer type and the hops added during brewing. Likewise, beer can be considered a dietary source of polyphenols and was found to be the main food contributor to hydroxybenzoic acid intake in the European Prospective Investigation into Cancer and Nutrition cohort study[56].

The phenolic concentration of commercial beers varies greatly, as it is show in **Table 2,** and depends on their extraction during maceration. During the brewing process, the phenolic concentration decreases in the protein precipitation and filtration processes, which are used to eliminate turbidity and bitterness. Moreover, during the wort boiling and fermentation, some polyphenols can undergo chemical changes such as isomerizations and decarboxylations, for example, hops used in brewing are particularly rich in xanthohumol (XN).However during the brewing process, XN isomerizes into isoxanthohumol (IX), resulting in beer having larger amounts of IX than XN[57]. These chemical changes can also produce aromatic volatile compounds. For instance, ferulic acid can be transformed to 4-vinilguaicol by decarboxylation per thermal decomposition[58].

Therefore, beer is a fermented beverage with a wide and varied phenolic profile and can be considered a dietary source of polyphenols. Further, hops polyphenols should be taken specially interest since they have demonstrated a powerful estrogenic activity, stronger than the well-known isoflavones and lignans[59].

Table 2. Beer polyphenols and their concentration found in regular beer. Source: Phenol-explorer (Database on polyphenols content in foods)[55]

	Phenolic compound	mean (min-max) in µg/100 mL
Flavonoids		
Chalcones	Xanthohumol	1.41 (0-3.4)
Flavanols	(+)-Catechin	110 (0-550)
	(-)-Epicatechin	60 (0-130)
	Procyanidin dimer B3	160 (0-360)
	Procyanidin trimer C2	30 (30-30)
	Prodelphinidin dimer B3	180 (0-450)
	Prodelphinidin trimer C-GC-C	20 (20-20)
	Prodelphinidin trimer GC-C-C	10 (0.01-10)
	Prodelphinidin trimer GC-GC-C	40 (40-40)
Flavanones	6-Geranylnaringenin	0.43 (0-1.1)
	6-Prenylnaringenin	2.6 (0-5.5)
	8-Prenylnaringenin	1.4 (0-2.1)
	Isoxanthohumol	40 (0-100)
	Naringin	0.75 (0-9)
Flavones	Apigenin	4.17 (0-50)
Flavonols	3,7-Dimethylquercetin	0.25 (0-2)
	Myricetin	0.67 (0-8)
	Quercetin	6.7 (0-40)
	Quercetin 3-O-arabinoside	0.58 (0-4)
	Quercetin 3-O-rutinoside	90 (0-490)
Isoflavonoids	Biochanin A	1.5 (0-10)
Phenolic acids		
Hydroxybenzoic acids	2,6-Dihydroxybenzoic acid	90 (3.9-250)
	2-Hydroxybenzoic acid	200 (0-660)
	3,5-Dihydroxybenzoic acid	30 (1.2-30)
	3-Hydroxybenzoic acid	30 (4.5-30)
	4-Hydroxybenzoic acid	960 (0-1680)
	Gallic acid	70 (0-700)
	Gallic acid 3-O-gallate	260 (180-370)
	Gentisic acid	30 (30-30)
	Protocatechuic acid	50 (0-80)
	Syringic acid	20 (0-90)
	Vanillic acid	70 (0-220)
Hydroxycinnamic acids	4-Caffeoylquinic acid	10 (0-20)
	5-Caffeoylquinic acid	80 (0-250)
	Caffeic acid	30 (0-50)
	Ferulic acid	260 (10-1410)
	m-Coumaric acid	20 (7.1-30)
	o-Coumaric acid	150 (10-170)
	p-Coumaric acid	100 (0-220)
	Sinapic acid	20 (0-130)
Hydroxyphenylacetic acids	4-Hydroxyphenylacetic acid	30 (0-140)
	Homovanillic acid	50 (2.4-50)
Other polyphenols		
Alkylmethoxyphenols	4-Vinylguaiacol	150 (0-410)
Alkylphenols	3-Methylcatechol	0.1 (0.1-0.1)
	4-Ethylcatechol	0.6 (0.6-0.6)
	4-Vinylphenol	4.53 (4.53-4.53)
Hydroxybenzaldehydes	Vanillin	20 (0-50)
Hydroxybenzoketones	2,3-Dihydroxy-1-guaiacylpropanone	3.4 (3.4-3.4)

Hydroxycoumarins	4-Hydroxycoumarin	110 (0-890)
	Esculin	20 (0-190)
	Umbelliferone	1.67E-29 (0-20)
Tyrosols	Tyrosol	320 (180-1180)
Other polyphenols	Catechol	1.1 (1.1-1.1)
	Pyrogallol	4.7 (4.7-4.7)

3.3. Alcohol

3.3.1. Alcohol, cardiovascular diseases and mortality

Diet plays a crucial role in the development of cardiovascular diseases, especially in atherosclerosis, since it can modify several cardiovascular risk factors. Numerous epidemiological studies have concluded that moderate alcohol consumption (up to 30 g alcohol/day for men and 20 g alcohol/day for women) protects against cardiovascular diseases development and even reduces cardiovascular mortality and all-cause mortality[60] (**Figure 6**).

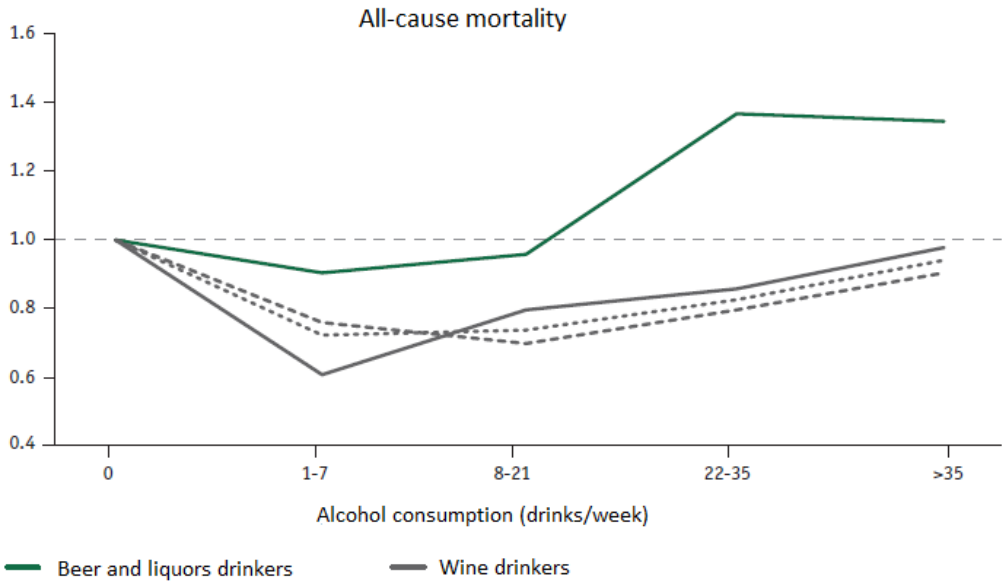

Figure 6. Relative Risk of all-cause mortality according to the alcohol consumption.
The relative risk is fixed at 1.00 in non-drinkers (<1 drink/week). All adjusted by age, sex, level of education, smoking, physical activity and body mass index. Source: Grønbaek *et al.* 2000[60].

Moderate consumption of alcohol, independently of the alcoholic beverage consumed, is associated with higher plasmatic concentrations of HDL-chl, apolipoprotein A1 (ApoA1) and adiponectin and lower concentrations of circulating fibrinogen, thus is associated with lower cardiovascular mortality[61]. However, not all alcoholic beverages have the same composition. Spirits are basically composed of alcohol and water, whereas fermented beverages contain

additional bioactive compounds, like polyphenols. Hence, beer and wine may confer additional protective effects. A meta-analysis published 15 years ago compared the wine and beer effects on cardiovascular risk. It was observed that both wine and beer consumption lower cardiovascular risk; however wine intake had a greater protective effect reducing the cardiovascular risk by 32%, while beer intake reduced the risk by 22%[62]. Nevertheless, in a most recent meta-analysis and systematic review performed by Costanzo *et al.* [63], it was observed that the protective effects of wine and beer were very similar and always greater than liquors and spirits. As it is shown in **Figure 7**, wine and beer curves have a "J" shape and they are practically overlapping. The maximum protection is observed at mean consumption of 25 g alcohol/day (260 mL of wine/day that is approximately a glass of wine a day or 625 mL of beer/day, which corresponds approximately to two cans of beer a day). This inverse association of wine and beer intake with incident cardiovascular events was not observed in liquors consumption.

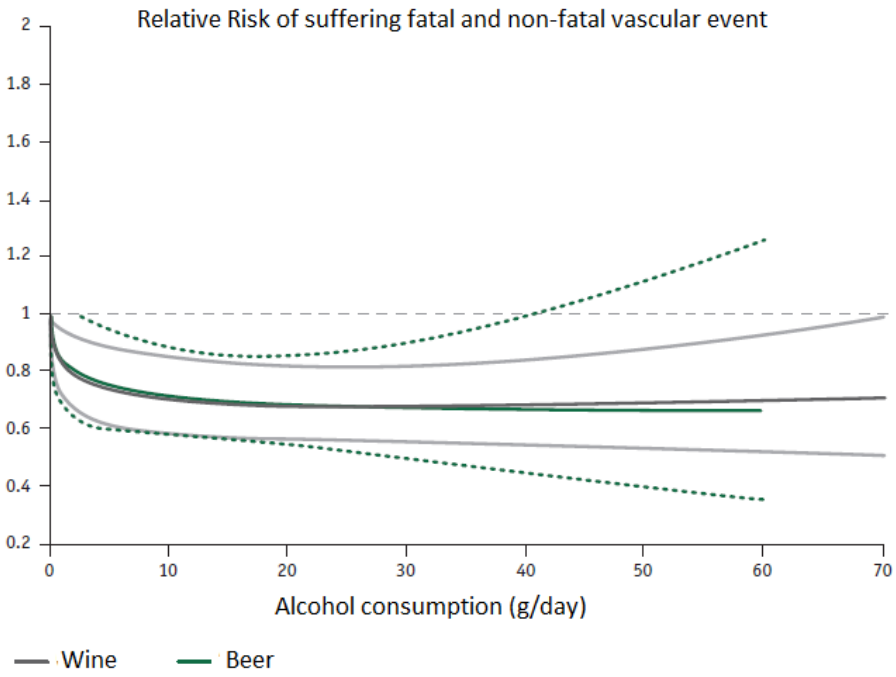

Figure 7. Relative Risk of incidence of cardiovascular event according to wine and beer consumption. Source: Costanzo *et al.* 2011[63]

Another two meta-analysis including 20 and 15 cohort studies[64,65], respectively, observed that moderate consumption of alcohol, independently of the alcoholic beverage consumed, had a protective effect against type II diabetes compared to abstainers or heavy drinkers (>60 g alcohol/day for men and >50 g alcohol/day for women). Demonstrating one more time the "U" or "J" shape association between alcohol and incident cardiovascular diseases or certain

cardiovascular risk factors. It is important to highlight that these protective effects of alcohol are only observed when the consumption is moderate. Higher intake of alcohol is associated with harmful effects on health. For example, in the Nurses's Health Study[66] higher consumption of 15 g alcohol/day was associated with a poorer glycemic control.

The effect of moderate alcohol consumption on the endothelial function and blood pressure is still controversial. In a multiethnic study, subjects who drank more than one glass/month and less than two glasses/day had better endothelial function measured by "flow mediator dilation" method[67]. It has also been observed that higher intake of 18 mL alcohol/day reverts the hypotensive effect of moderate alcohol consumption[68]. However, some other studies suggest that alcohol consumption has no association with blood pressure and does not increases the incidence of hypertension[69,70].

In conclusion, several epidemiological studies associate moderate alcohol consumption with a beneficial effect on cardiovascular system; however, many of them do not discriminate between types of alcoholic beverage consumed, hence recommendations on which alcoholic beverage is more beneficial to consume is still controversial. Moreover, we must be cautious interpreting epidemiological data due to the potential confounding factors to be considered in the studies and the fact that cohort studies cannot demonstrate a cause-effect relation, which can only be demonstrated in controlled intervention clinical trials.

3.3.2. Noxious effects of excessive alcohol consumption

Excessive and binge drinking of alcohol is unquestionable harmful and is a risk factor of several diseases, both physical and social, such as hypertension, acute myocardial infarction, cardiomyopathies, cardiac arrhythmia, hepatic cirrhosis, pancreatitis, neuropathies, encephalopathies, sudden death syndrome, violence, suicide, and unintentional injuries.

According to the World Health Organization report 2011, the harmful consumption of alcohol caused the 3.8% of the global deaths in 2004, and more than 50% of them were due to CVD, hepatic cirrhosis and cancer.

Moreover, consumption of alcohol at any dose is not recommended for children, adolescents, pregnant women, individuals at risk of alcoholism, individuals with cardiomyopathy, cardiac arrhythmias, depression, or liver and pancreatic disease, or during performing actions that require concentration, skill or coordination.

3.4. Nutritional Biomarkers and metabolomics

3.4.1. Definition, classification and importance

According to Biomarkers Definitions Working group, a biomarker is "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention"[71]. Further, the World Health Organization has defined a biomarker as "almost any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical, or biological. The measured response may be functional and physiological, biochemical at the cellular level, or a molecular interaction"[72] . Thus, the definition takes into account not just incidence and outcome of disease, but also the effects of treatments, interventions, and even unintended environmental exposure, such as chemicals or nutrients. Examples of biomarkers ranges from blood pressure measures and basic chemistries to more complex laboratory test of different biological samples.

In molecular epidemiology, biomarkers are classified in three categories:

- Biomarkers of exposure: Chemical compounds or metabolites that can be measured in body fluids after the exposure of the chemical in the organism. Thus, the biomarker has the ability to identify if the exposure has occurred. For example, levels of mercury in the body due to fish consumption.
- Biomarkers of effect: Quantifiable changes that an individual undergoes, which may indicates an exposure to a compound or a resulting health effect. For example, markers for early pathological changes in complex disease developments, such as mutations and preneoplastic lesions. A more precise example could be acetylcholinesterase inhibition by exposure to organophosphate pesticides.
- Biomarkers of susceptibility: Indicators of an elevated sensitivity to the effects of an environmental agent that can be objectively measured in a biological system or a sample. They are used to identify "at-risk" individuals. Risk may be either acquired (e.g. indicator of a disorder that make people more susceptible to an environmental exposure) or inherited (as indicated by genetic markers).

Nutritional biomarkers belong to the biomarkers of exposure category and are indicators of dietary exposure. The more common nutritional biomarkers used are compounds found in food and their metabolites, although physical properties such as stable isotope ratio are also suitable. Depending on the relationship between intake and biomarker, they are divided into three main classes[73]:

- Recovery biomarkers: They are based on total excretion of the marker in a defined time period. Only a few recovery markers have been described, for example total urinary nitrogen and potassium.
- Concentration biomarkers: They are only based on the concentration of the respective marker and they do not provide information on the physiological balance of intake and excretion. For this reason, they cannot be used to estimate absolute intake, although, since they are correlated with intake, they can be used to rank intake of specific nutrients.
- Predictive biomarkers: Predictive biomarkers have been proposed by Tasevska *et al.*[74] to describe biomarkers with incomplete recovery but stable and time-dependent high correlation with intake. For example, urinary sucrose and fructose as a marker of sugar intake[75].

Nutritional biomarkers are important in nutritional epidemiology since this science relies on accurate dietary information to investigate associations between diet and disease risk. Most techniques used in assessing the diet followed by individuals are based on self-reporting, which is liable to systematic bias by factors such as age, gender, social desirability and approval[7,8]. Food-frequency questionnaires (FFQ) are known to over-report fruit and vegetable intake[76] and obese people are known to under-report total energy, protein and sugar intake[77,78]. Nutritional biomarkers can provide additional exposure assessments with unrelated measurement error that can be used in statistical models to correct the self-reporting errors. Moreover, they provide an integrated measure of the bioavailability and metabolism of the component. And, data on nutrient intake (for example, polyphenol intake) based on biomarkers correlates better with clinical outcomes (such as blood pressure) than data obtained by FFQ[79]. Furthermore, nutritional biomarkers are also very useful in clinical trials for monitoring compliance with administered interventions.

Biomarkers are, in particular, useful in estimating intake of specific bioactive compounds such as phytoestrogen[80]s and polyphenols[81] since food composition databases lack of information of such compounds and the large variability of those within foods. However, this also represents a key limitation of nutritional biomarkers; it is very difficult to identify the source of these compounds using biomarkers alone, unless the compound is specific to only a certain type of food. Another limitation of biomarkers is the difficulty in assessing intake of food groups, for example, fruits and vegetables. Moreover, intra and inter-individual differences in absorption and metabolism also affect the measured concentration of the biomarker in the biological sample since differences in the metabolism affect circulating levels of the biomarkers and their analysis. Several biomarkers, like polyphenols, undergo extensive metabolism by intestinal

microbiota and liver (phase-II metabolism). Thus, the analytical methods used for the biomarkers analysis often fail to detect most of the metabolites, introducing an additional bias. A further limitation is their short half-life in the organism. For example, most of phenolic compounds are excreted within less than 24 hours, providing information of only the intake of the day before or previous days. Lipophilic biomarkers, like carotenoids, provide information of longer periods as their half-life is up to 100 days[82], however this period is still shorter than the time covered by FFQ.

3.4.2. Validation of nutritional biomarkers

An effective biomarker of food intake should be specific to the dietary component of interest, sensitively reflect changes in food intake by its concentration in the biofluid, have an adequate half-life in the biofluid, provide good correlation between excretion and exposure, and have a robust quantification method[83,84].

New potential biomarkers need to be validated in order to verify they fulfil the criteria mention above. Ideally, biomarkers should be validated in two stages[73]:

- In dose-response controlled clinical trials to identify the range of intake in which the biomarker is reliable.
- In free-living populations to evaluate the suitability of the biomarker in a habitual diet.

The validation of the biomarker in controlled clinical trials also provides information about potential limitations such as range of intake and interactions with other foods. However, most biomarkers are validated using self-reported intake data only instead of full validation using control clinical trials.

3.4.3. LC-based techniques for biomarkers discovery and metabolomics

The development of a biomarker consist in two parts: discovery and validation. This section will be focus on biomarker discovery using liquid-chromatography coupled to mass spectrometry (LC-MS) techniques.

LC-MS techniques for biomarker discovery can be divided into two approaches:

- Hypothesis driven approach (or targeted analysis): Targeted analysis focus on identifying and quantifying selected compounds or class of compounds. The analytical procedure includes the identification and absolute quantification of the selected analyte in the sample. This approach requires knowledge of food constituents as well as the bioavailability and metabolism. Usually, the most common metabolites are chosen as candidate biomarker and analytical methods are developed specifically for the analysis

of the potential biomarker. Most of the biomarkers reported have been developed using this method.

- Discovery-driven approach (or untargeted analysis): Untargeted analysis involves rapid analysis, usually semi-quantitative, of a large number of different metabolites in order to identify a specific metabolite profile that characterize a given sample. This approach has recently become more important with the onset of metabolomics techniques. In this approach, candidate biomarkers are identified in samples from controlled clinical interventions using multivariate statistics techniques, such as discriminant analysis. The advantage of this approach is that a much less priori information of the food matrix is needed as the candidate biomarker is identified using statistical analysis. However, it requires very carefully controlled dietary intervention and sample collection protocols since the multivariate techniques are very sensitive to sample differences. Besides, all instrumental conditions such as equipment calibration, column performance, sensitivity or injector operation, among others, need to be well controlled in order to ensure no variations across sample runs to avoid false results.

Both approaches are used in biomarkers discovery and metabolomics. Metabolomics is the endpoint of the ‘‘omics cascade’’ and is the closest to phenotype (**Figure 8**). The aim of metabolomics is to profile all metabolites found in a sample to investigate the effect of a particular stimulus on metabolic pathways[85]. Thus, metabolome analysis covers the identification and quantification of all intra- and extracellular metabolites with molecular mass lower than 1800 Da.

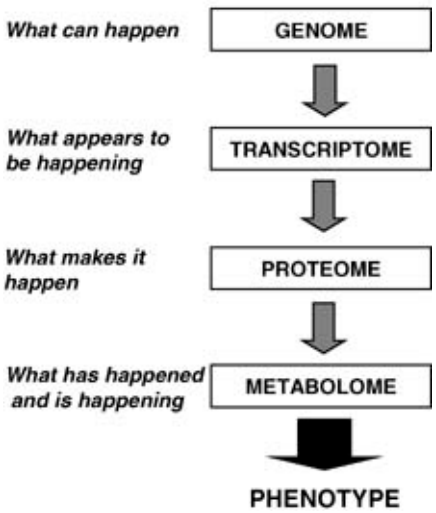

Figure 8. The "omics" cascade. Source: Dettmer *et al.* 2006[86]

The food metabolome is defined as the part of the human metabolome derived from the digestion and biotransformation of foods and their constituents[87]. The food metabolome is extremely complex and varies according to the diet. Diet influences both endogenous metabolome and food metabolome. The endogenous metabolome includes all metabolites of the body and its variations show novel metabolic effects of the diet that may affect human health. Furthermore, humans are exposed to a variety of "chemical environments" associated with the food we consume. Thus, the human metabolome is not just a single entity but consists of several components (**Figure 9**), including the endogenous metabolome, the food metabolome and other xenobiotics from drugs or environmental chemicals[87] and also food packaging.

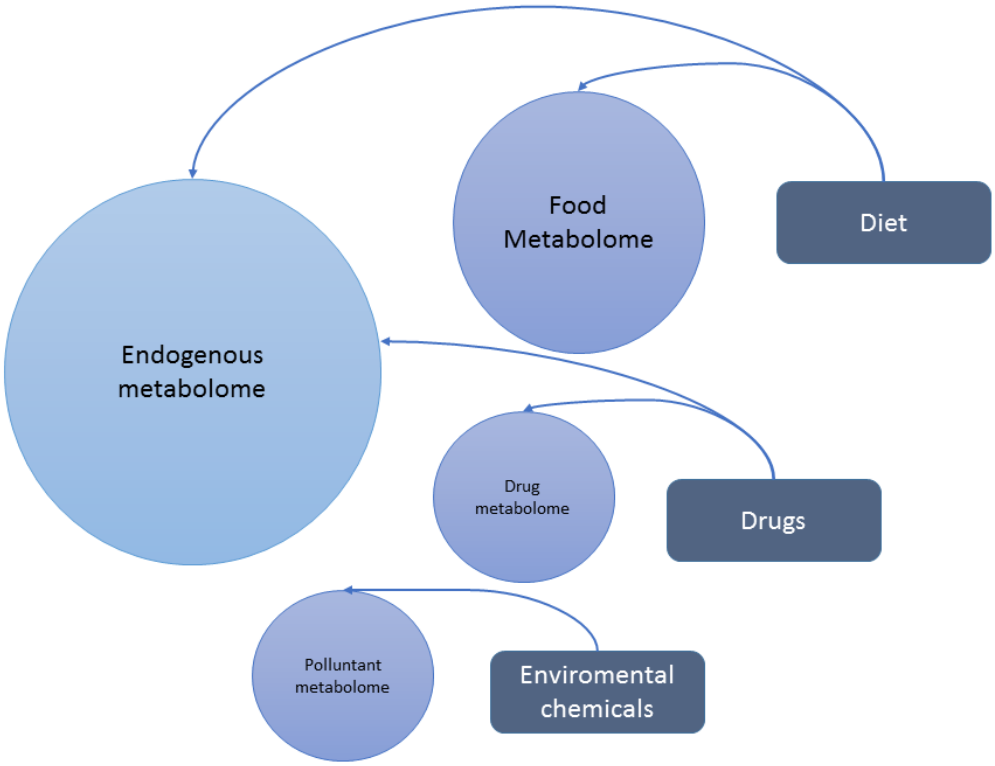

Figure 9. The human metabolome

Therefore, the whole metabolome consists of extremely diverse chemical compounds from ionic inorganic species to hydrophilic carbohydrates, volatile alcohols and ketones, amino and non-amino organic acids, hydrophobic lipids, and complex natural products. This complexity makes impossible to determine the complete metabolome in a single analysis and it can be studied using different samples preparation protocols and analytical techniques, either more specific for a group of analytes (targeted analysis) or more general to the whole sample (untargeted

analysis). So, unlike the other "omics" techniques such as genomics and transcriptomics, there is no single instrument platform that currently can analyze all metabolites.

Mass spectrometry (MS) analysis is widely used in metabolomics and biomarker discovery due to its high sensitivity, selectivity, high-throughput to confirm the identity of the components present in the biological sample and the detection of unknown compounds. Moreover, the combination with separation techniques, such as chromatography, increases the capability of the chemical analysis of highly complex biological samples, such as urine and plasma, due to metabolite separation in a time dimension, provides isobar separation, and delivers additional information on the physicochemical properties of the metabolites. LC-MS techniques revolutionized the bioanalysis of small molecules in the late 80's[88]. Nowadays, MS is the most important detection technique in bioanalysis. Especially, after the introduction of ESI (electrospray ionization) and APCI (atmospheric-pressure chemical ionization).

A mass spectrometer is typically composed of three major parts: ion source, mass analyzer, and detector. Mass analyzers can be categorized as: quadrupole, ion trap (IT), time-of-flight (TOF), Orbitrap, and Fourier transform ion cyclotron (FTICR). Hybrid or tandem mass spectrometers refer to the combination of two or more analyzers, for example triple quadrupole (QqQ), quadrupole TOF (QqTOF) or quadrupole ion trap (QIT).

The ionization technique of choice for LC-MS based metabolic profiling is ESI due to its "soft ionization" capability that produces a large number of ions through charge exchange in solution[86,89].APCI can be used as complimentary to ESI for the analysis of non-polar and thermally stable compounds such as lipids. Hereby, instruments with dual ionization capabilities, such as ESI and APCI, have gradually become the trend since it increases the coverage of the metabolome.

LC-MS for targeted analysis

LC-MS provides a unique combination of resolving power, sensitivity, and specificity that offers the possibility to identify target metabolites in a very complex mixture. Low resolution mass spectrometry (LRMS), such as QqQ or QIT, is mostly used for targeted analysis. QqQ is usually set in multiple reaction monitoring mode (MRM). In MRM experiments, the precursor ion is dissociated to fragment ions but only a selected fragmented ion is chosen for detection. The two-stage ion selection procedure results into a sensitive measurement that is specific to both molecular weight (precursor ion) and structure (fragment ion) of a given analyte. MRM is the best mode to measure the real concentration of several metabolite in a single run and is performed by using different quantitation methods such as standard addition (where the

standard is added to a blank sample matrix constructing a calibration curve) or isotopic dilution (where the use of isotopically labelled compounds eliminates the ion suppression effect due to sample matrix). Thus, QqQ-based LC-MRM-MS/MS has become the quantitative approach of choice in targeted metabolic analysis[90,91].

The main advantage of targeted approach is that the chemical properties of the selected compounds are known, so analyte extraction methods can be developed to recover the maximum amount of analytes in complex sample and to reduce matrix effects and interferences. For instance, selective extraction methods that include a clean-up step like SPE can be used. Another advantage of targeted analysis is that chromatographic conditions such as column election, mobile phase pH and elution gradient can be developed. Moreover, MS working parameters like energy collision, temperature of the ion source, polarity, and other potential parameters and can also be optimized using pure standards in infusion experiments.

There are several published works that used targeted metabolomics and it has been widely used to analyze polyphenols and their related metabolites in biological samples. Here, only a few examples are described. De Ferrars *et al*. 2014 quantified a total of 28 and 21 anthocyanidins and their related metabolites (including conjugates and phenolic acids) in urine and plasma, respectively, in post-menopausal women after an acute ingestion of elderberry extract containing 500 mg of anthocyanidins[92]. Their analysis was performed using HPLC coupled to a hybrid triple quadrupole lineal ion trap and samples were previously extracted using SPE. Pimpão *et al.* 2015 used a LC-ESI-triple quadrupole mass spectrometer to quantify phenolic sulfates in plasma after an acute ingestion of a fruit purée composed of blueberries, blackberries, raspberries, strawberries and crowberries[93]. Martinez-Huélamo *et al.* 2016 evaluated the oil addition in tomato sauces on the bioavailability of tomato polyphenols. For this purpose, they quantified 10 phenolic compounds in plasma and 93 in urine, including flavanones, hydroxycinnamic acids, flavonols, and their related microbiota metabolites using and HPLC-ESI-MS/MS approach in a triple quadrupole mass spectrometer[94].

LC-MS for untargeted analysis

The main objective of untargeted metabolomics approach is to detect as many metabolites as possible in a given sample to identify compounds that are dysregulated due to a particular biological condition. LC coupled to high-resolution mass spectrometry (HRMS) can detect and identify unknown compounds that are present in a biological matrix without any prior knowledge of their exact chemical structure. Thus, LC-MS is an important tool for metabolite profiling. The capabilities of LC-MS are further increased with the multiple-stage mass

spectrometry experiments (MS^n) introduced to acquire product ion spectra (where *n* is the number of product ion stages), especially for structure elucidation.

In order to obtain a broad coverage of the metabolome, ionization must be performed in both positive and negative mode[86] under a scan range of *m/z* 50–1000 to maximize metabolome coverage and a previous optimization using a quality control pool of representative standards (i.e. showing different polarities) must be done to experimental LC-MS conditions.

High-resolution mass spectrometers, such as FTICR, Orbitrap, and TOF, provide accurate mass measurements that enable metabolite identification. They also acquire highly resolved and accurate MS/MS spectra, which helps to the metabolite identification and confirmation. This is achieved through ion fragmentation by collision-induced dissociation (CID) in either quadrupole based tandem in-space instruments, such as triple quadrupole and QqTOF, or ion trap-based tandem in-time instruments, such as quadrupole-ion trap, ion trap-TOF, LTQ-Orbitrap and LTQ-FTICR. Determination a possible molecular formula with errors < 1-2 ppm, matching between theoretical and experimental isotopic distribution, and comparison of retention time and MS^2 spectra with a pure standard or with bibliographic data are the main steps to identify a compound.

Most metabolomics studies use a separation step before MS analysis. HPLC separates compounds of a wide range of polarity through isocratic elution or gradient elution. Acetonitrile and methanol are the most common organic solvents used[89] due to their compatibility with MS. Gradient elution generally performs a faster analysis, narrower peaks and similar resolution compared to isocratic elution[95]. The most common chromatographic columns used in metabolomics are reverse phase (RP) C_{18} columns since they generate reproducible data for a large set of metabolites (non- and moderately polar compounds). However, in order to cover a wider range of polarity and determine polar compounds like sugars, amino acids, vitamins, carboxylic acids, and nucleotides or non-polar compounds such as acylcarnitines, phosphatidylcholines, sphingolipids and triacylglycerides other chromatographic modes like Hydrophilic-Interaction Chromatography (HILIC) or C_8 columns need to be used[96]. Contrepois *et al.* 2015 reported that combining HILIC and RP-LC, the metabolome coverage of urine and plasma increased by 44% and 108%, respectively, compared to RP alone[97].

In MS-based investigation, metabolite profiling are described by *m/z* (mass-to-charge ratio) values and the corresponding intensities of detected ions. If the analysis is followed by a separation step, retention times are also used to index metabolites. Thus, features are defined as a molecular entity with a unique *m/z* value and retention time. Features' intensities are used as a semi-quantitative data and are exported for sample classification using multivariate analysis or inference statistical analysis using univariate tools. In order to define these features, once all

samples are acquired, the raw LC-MS data must be converted into a peak list defined by *m/z* value, retention time and intensity using multiple pre-processing steps:

1. Filtering: removes the noise and contaminants from LC-MS data
2. Baseline correction: estimate the low-frequency baseline and subtract the estimated baseline from the raw signal
3. Peak detection: converts the raw continuous data into centroided discrete data so each ion is represented as a peak
4. Peak matching and retention time alignment: enables the comparison of LC-MS data across samples. In case that a slight variation of retention time is observed. A maximum deviation must be defined
5. Ion annotation: groups together ions that are likely to originate from the same compound.
6. Normalization of peak intensities to reduce the systematic variation of LC-MS data.

There are currently several algorithms and softwares that performed these pre-processing steps, including open-source based algorithms such as XCMS[98], MZmine[99] and MetAlign[100] and vendor software like Agilent Mass Profiler Professional and Waters MerkerLynx.

The pre-processed results can be exported for further statistical analysis, such as univariate and multivariate statistical analysis. The univariate approach assesses the statistical significance of each peak separately. The more common used univariate techniques are t-test, fold-change analysis (volcano plot), Wilcoxon rank-sum test, and analysis of variance (ANOVA), among others. Due to thousands of metabolites are simultaneously measured, the multiple hypothesis testing results in a high chance of false discovery even with a small *p*-value threshold. The false discovery rate (FDR) is used to estimate the chance of false discovery in a given test statistics threshold. A *q*-value for each peak is estimated which is the minimum FDR at which that peak is called significant.

Multivariate analysis considers the combinatorial effect of multiple variables. It is further categorized as unsupervised and supervised techniques. Unsupervised are methods that identify hidden structures in the data without knowing the class labels. One of the most popular unsupervised techniques in the LC-MS-based metabolomic approaches is principal component analysis (PCA). In contrary to unsupervised techniques, supervised analysis use the class label information to construct a model in order interpret the LC-MS data. The most common supervised technique is Partial least square-discriminant analysis (PLS-DA). PLS-DA finds the projection direction which gives the largest covariance between the data and the labels. It is capable to find the discriminant metabolites in the pre-defined groups[101].

The major challenge in metabolomic studies is the identification of metabolites. At present, metabolite identification is mainly achieved through mass-based search in online databases using the *m/z* values followed by manual verification and confirmation using MS/MS spectra. The most common databases used are the Human Metabolome Database[102] and METLIN[103]. The search is performed using a tolerance range, usually 5 ppm, which retrieves a list of putative identified compounds. However, the mass-based search can rarely provide unique identification for the ions of interest due to several reasons. One reason is even using a low mass accuracy, like 1 ppm (which most HRMS platforms cannot achieve), it is still no sufficient for unambiguous identification owing to the presence of compounds with extremely similar molecular weights. Secondly, mass-based identification cannot discriminate isomers, which have the same elemental composition. Finally, all metabolite databases have a limited coverage of the entire metabolome. It has been estimated that generally, less than 30% of the detected ions in a LC-MS experiment can be uniquely identified, resulting most of the ions unknowns or with multiple putative identification. To verify the mass-based search results, putative identifications are subjected to MS or tandem MS experiments. Moreover, putative compounds may be further confirm using pure standards comparing the retentions times and the MS spectra. The most limiting factor of verification is that it is often costly and time consuming, for example, molecular ion can have more than 100 putative identifications, which make manual verifications extremely laborious.

Although discovery-driven approach is rather new, several nutritional metabolomics works have been published. For example, Cho *et al*. 2016 were able to discriminate obese from normal-weight adolescents according to their urinary metabolomics profile by LC-QqTOF[104]. In this work, they found that seven endogenous metabolites were distinguished in the obese group including 4-hydroxybenzaldehyde, hippuric acid, 4-sulfobenzyl alcohol, N,N-dimethylsafingol, docosanoic acid, 4α-hydroxymethyl-5α-cholesta-8-en-3β-ol and 12-oxo-20-carboxy-leukotriene B4. In another work, Jacobs *et al.* 2012 evaluated the effect of red wine and grape polyphenols on the urine and plasma metabolome[105]. Syringic acid, 3-hydroxyhippuric acid, pyrogallol, 3-hydroxyphenylacetic acid, and 3-hydroxyphenylpropionic acid were found to be the strongest urinary markers of red wine and grape juice intake. Moreover, red wine and grape juice consumption had a mild impact of the endogenous metabolome by changing amino acids from tyrosine and tryptophan, reducing microbial metabolites *p*-cresol sulfate and 3-indoxylsulfuric acid and increasing in indole-3-lactic acid and nicotinic acid. Lastly, Hanhineva *et al.* 2015 used non-targeted LC-MS approach to discover urinary biomarkers of whole grain rye[106]. They found that Hydroxyhydroxyphenyl acetamide sulfate, 3,5-dihydroxyphenylpropionic acid sulfate,

caffeic acid sulfate, and hydroxyphenyl acetamide sulfate had the greatest potential as intake biomarkers of whole grain.

4. RESULTS

This section states all the works performed during this doctoral thesis.

The results are collected in eight papers; five of them have been published in journals included in the Science Citation Index. All articles are published in journals ranked in the first quartile, except for one article that is published in Nutrition, metabolism and cardiovascular diseases which belongs to the second quartile. The remaining three papers have been submitted and are under revision to reference journals.

A brief summary containing objective of the study, methodology, results and conclusion is included prior each publication.

This section is further organized in three blocks:

- Identification of beer polyphenols
- Biomarkers of beer consumption. Validation of Isoxanthohumol as an accurate biomarker, discovery of potential new biomarkers of beer consumption and metabolomics
- Beer, beer polyphenols and cardiovascular diseases

4.1. Identification of beer polyphenols

4.1.1. Publication 1. A comprehensive characterization of beer polyphenols by high-resolution mass spectrometry (LC-ESI-LTQ-Orbitrap-MS).

Paola Quifer-Rada, Anna Vallverdú-Queralt, Miriam Martínez-Huélamo, Gemma Chiva-Blanch, Olga Jáuregui, Ramon Estruch, Rosa Lamuela-Raventós. *Food Chemistry* 2015;169:336-43.

Summary:

Beer is the second most consumed alcoholic beverage in Europe according to the European Spirits Organization. Beer contains carbohydrates, minerals (potassium, magnesium), vitamins (niacin, riboflavin, folate, cobalamin, pyridoxine) and amino acids. Additionally, beer contains polyphenols which about 70-80% come from malt, and the remaining 30-20% come from hops. The European Prospective Investigation into Cancer and Nutrition cohort study estimated that beer is the main food contributor to hydroxybenzoic acid intake. Thus, beer consumption may be a good source of polyphenols. However, although various phenolic compounds have been described in beer using different analytical detectors, a comprehensive identification of its phenolic profile by high-resolution mass spectrometry has been lacking. In this work, liquid chromatography coupled with an electrospray ionization hybrid linear ion trap quadrupole Orbitrap mass spectrometry technique has been used for an accurate identification of beer polyphenols. Four types of beer were used for the identification of the phenolic compounds present in beer: lager, Pilsen, Märzenbier and non-alcoholic beer. In order to improve sensitivity, a solid phase extraction step was applied. Mass spectra were acquired in profile mode and the most intense ions detected during full scan MS triggered data-dependent scanning. Phenolic compounds were identified by generating the molecular formula using accurate mass with some restrictions (C=30, H=100, O=15), and matching with the isotopic pattern. This molecular formula was then identified using the Dictionary of Natural Products (Chapman & Hall/CRC). The phenolic compounds were further confirmed by comparisons with pure standards whenever possible as well as with the literature

A total of 47 phenolic compounds were identified using high mass accuracy and confirmed by MS^2 experiments, including simple phenolic acids, hydroxycinnamoylquinics, flavanols, flavonols, flavones, alkylmethoxyphenols, alpha- and iso-alpha-acids, hydroxyphenylacetic acids and prenylflavanoids. As far as we know, 7 of these compounds are described in beer for the first time: feruloylquinic acid, caffeic acid-*O*-hexoside, coumaric acid-*O*-hexoside, sinapic acid-*O*-hexoside, catechin-*O*-dihexoside, kaempferol-*O*-hexoside, and apigenin-*C*-hexoside-pentoside.

No differences in the phenolic profile were observed among the four types of beer, except for Märzenbier, which lacked catechin-*O*-hexoside.

Food Chemistry 169 (2015) 336–343

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

A comprehensive characterisation of beer polyphenols by high resolution mass spectrometry (LC–ESI-LTQ-Orbitrap-MS)

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ARTICLE INFO

Article history:
Received 9 April 2014
Received in revised form 20 June 2014
Accepted 21 July 2014
Available online 10 August 2014

Keywords:
Beer
Polyphenols
LTQ-Orbitrap
Phenolic acids
High resolution mass spectrometry

ABSTRACT

Beer is the second most consumed alcoholic beverage in Europe and shown by the European Prospective Investigation into Cancer and Nutrition cohort study to be the main food contributor to hydroxybenzoic acid intake. About 70–80% of the total polyphenol content in beer comes from malt, and the remaining 30–20% from hops. In this work, liquid chromatography coupled with an electrospray ionization hybrid linear ion trap quadrupole Orbitrap mass spectrometry technique has been used for an accurate identification of beer polyphenols. 47 phenolic compounds were identified using high mass accuracy and confirmed by MS^2 experiments, including simple phenolic acids, hydroxycinnamoylquinics, flavanols, flavonols, flavones, alkylmethoxyphenols, alpha- and iso-alpha-acids, hydroxyphenylacetic acids and prenylflavonoids. As far as we know, 7 of these compounds have been recognised in beer for the first time: feruloylquinic acid, caffeic acid-*O*-hexoside, coumaric acid-*O*-hexoside, sinapic acid-*O*-hexoside, catechin-*O*-dihexoside, kaempferol-*O*-hexoside, and apigenin-*C*-hexoside-pentoside.

1. Introduction

Beer is the second most consumed alcoholic beverage in Europe, accounting for 37% of the total EU alcohol consumption, according to the European Spirits Organization. The average beer consumption per capita in Europe in 2009-2011 was 72.8 L. Beer contains carbohydrates, minerals (potassium, magnesium), vitamins (niacin, riboflavin, folate, cobalamin, pyridoxine) and amino acids. Additionally, beer contains polyphenols of which about 70–80% come from malt, and the remaining 30–20% come from hops (De Keukeleire, 2000). The main phenolic compounds are hydroxybenzoic acids, cinnamic acids, such as ferulic acid, and flavonols (Gerhäuser, 2005). Hop polyphenol content depends on the type of beer and the quantity of hops added during production. Furthermore, during the brewing process and fermentation, some polyphenols undergo chemical changes, such as decarboxylation and isomerization. Beer constitutes a good source of polyphenols and was found to be the main food contributor to hydroxybenzoic acid intake in the European Prospective Investigation into Cancer and Nutrition cohort study (Zamora-Ros et al., 2013).

Although various phenolic compounds have been found in beer using different detectors, such as the coulometric array (Floridi, Montanari, Marconi, & Fantozzi, 2003; Jandera et al., 2005; Rehová, Skeríková, & Jandera, 2004), electrochemical (Madigan, McMurrough, & Smyth, 1994; Montanari, Perretti, Natella, Guidi, & Fantozzi, 1999; Nardini & Ghiselli, 2004; Piazzon, Forte, & Nardini, 2010; Vanbeneden, Delvaux, & Delvaux, 2006), photodiode array (Bartolomé, Peña-Neira, & Gómez-Cordovés, 2000), ultraviolet-visible spectrophotometry (Arts, van de Putte, & Hollman, 2000; McMurrough, Madigan, & Smyth, 1996) and low resolution mass spectrometry (Ceslova, Holcapek, Fidler, Drstickova, & Lisa, 2009; Vanhoenacker, De Keukeleire, & Sandra, 2004), a comprehensive identification of its phenolic profile by high resolution mass spectrometry is still lacking. High-resolution/accurate mass measurement mass spectrometry techniques have proven to be a reliable tool for the structural elucidation of unknown compounds in complex samples. In this context, linear ion trap quadrupole-Orbitrap-mass spectrometry (LTQ-Orbitrap-MS) provides single-stage mass analysis that supplies molecular weight information, two-stage mass analysis (MS/MS) and multi-stage mass analysis (MS^n) that provides structural information. Exact mass

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http://dx.doi.org/10.1016/j.foodchem.2014.07.154
0308-8146/

measurements and molecular formula assignment are indispensable for the characterisation of polyphenols. Moreover, accurate mass measurement of the product ions facilitates the elucidation of unknown compounds.

The aim of this work was to identify the full range of polyphenols found in beer. Therefore, a solid-phase extraction procedure was applied in order to increase sensitivity and lower the matrix effect. High mass accuracy was used to identify 47 phenolic compounds, confirmed by product ion scan experiments and high mass accuracy of the fragments. To our knowledge, 7 phenolic compounds are reported in beer for the first time.

2. Materials and methods

2.1. Chemicals and reagents

Gallic, caffeic, protocatechuic, ferulic, chlorogenic, sinapic, *p*-coumaric, vanillic and protocatechuic acids, quercetin-3-*O*-glucoside, catechin and epicatechin (97–99% purity, all) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Isoxanthohumol, 8-prenylnaringenin and xanthohumol (97–99% purity) were purchased from Enzo Life Science (Lausen, Switzerland). Methanol (MeOH) and acetonitrile (MeCN) of HPLC grade were obtained from Sigma–Aldrich (St. Louis, MO, USA). HPLC grade acetic acid, formic acid and sodium acetate were purchased from Panreac Quimica S.A (Barcelona, Spain). Ultrapure water (MilliQ) was generated by the Millipore System (Bedford, USA). SPE cartridges and Oasis MAX 96-well plate 30 μm (30 mg) were obtained from Waters (Milford, MA, USA).

2.2. Samples

Four types of beer were used for the identification of the phenolic compounds present in beer: lager, Pilsen, Märzenbier and non-alcoholic beer. All beers were produced in Spain and were obtained from the market.

2.3. Sample preparation

Samples were prepared in a darkened room with a red safety light to avoid analyte oxidation. Beer foam was removed by ultrasonication and agitation using a magnetic stirrer. Then, in order to improve sensitivity, a solid-phase extraction step was applied following the method developed by Medina-Remón et al. for urine samples (Medina-Remon et al., 2009) and then used and validated in tomato samples (Vallverdu-Queralt, Jauregui, Medina-Remon, & Lamuela-Raventos, 2012) with minor modifications. Briefly, beer ethanol was evaporated under nitrogen flow. Oasis® MAX cartridges were activated with 1 mL of methanol and subsequently 1 mL of sodium acetate 50 mM pH 7. Then 1 mL of dealcoholized beer acidified with 34 μL of hydrochloric acid at 35% was loaded into the cartridges. Cartridges were rinsed with sodium acetate 50 mM pH 7.0 containing 5% methanol. Polyphenols were eluted with 1800 μL of methanol with 2% formic acid. The eluted fractions were evaporated under nitrogen flow, and the residue was reconstituted with water (0.1% formic acid) up to 100 μL and filtered through a 13 mm, 0.45 μm PTFE filter into an insert-amber vial for HPLC analysis. Samples were stored at −20 °C until analysis. In order to prove that the beer matrix did not influence recovery of the analytes, a recovery assay was performed using 12 representative beer polyphenols (4-hydroxybenzoic, caffeic acid, catechin, epicatechin, chlorogenic, ferulic acid, kaempferol-*O*-glucoside, *p*-coumaric acid, protocatechuic acid, quercetin-3-*O*-glucoside, sinapic and vanillic acids). The recoveries of the polyphenols in beer ranged from 78.3% ± 6.6% and 113.5% ± 8.6%.

2.4. LC–high resolution mass spectrometry and experimental conditions

An LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Hemel Hempstead, UK) equipped with an ESI source working in negative mode was used for accurate mass measurements. Mass spectra were acquired in profile mode with a setting of 30,000 resolution at *m*/*z* 400. Operation parameters were as follows: source voltage, 4 kV; sheath gas, 20 (arbitrary units); auxiliary gas, 10 (arbitrary units); sweep gas, 2 (arbitrary units); and capillary temperature, 275 °C. Default values were used for most other acquisition parameters (FT Automatic gain control (AGC) target $5 \cdot 10^5$ for MS mode and $5 \cdot 10^4$ for MS^n mode). Beer samples were analysed in full scan mode at a resolving power of 30,000 at *m*/*z* 400 and data-dependent MS/MS events acquired at a resolving power of 15,000. The most intense ions detected during full scan MS triggered data-dependent scanning. Data-dependent scanning was carried out without the use of a parent ion list. Ions that were not intense enough for a data-dependent scan were analysed in MS^n mode with the Orbitrap resolution also set at 15,000 at *m*/*z* 400. An isolation width of 100 amu was used and precursors were fragmented by collision-induced dissociation C-trap (CID) with a normalised collision energy of 35 V and an activation time of 10 ms. The mass range in FTMS mode was from *m*/*z* 100 to 1000. The data analysis was achieved using XCalibur software v2.0.7 (Thermo Fisher Scientific). An external calibration for mass accuracy was carried out before the analysis.

Liquid chromatography analysis was performed using an Accela chromatograph (Thermo Scientific, Hemel Hempstead, UK) equipped with a quaternary pump, a photodiode array detector (PDA) and a thermostated autosampler. Chromatographic separation was accomplished with a Phenomenex Luna C_{18} column 50 × 2.0 mm i.d., 3 μm (Phenomenex, Torrance, CA, USA). Gradient elution of analytes was carried out with water/0.1% formic acid (solvent A) and acetonitrile/0.1% formic acid (solvent B) at a constant flow rate of 0.4 L/min, and the injection volume was 5 μL. A non-linear gradient was applied: 0 min, 2% B; 0–2 min, 8% B; 2–12 min, 20% B; 12–13 min, 30% B; 13–14 min, 100% B; 14–17 min, 100% B; 17–18 min, 2% B and the column was equilibrated for 7 min to initial conditions.

3. Results and discussion

3.1. General

The data-dependent scan experiment was very useful for the identification of unknown phenolic compounds since it provides high resolution and accurate mass product ion spectra from precursor ions that are unknown beforehand within a single run. Combining data-dependent scan and MS^n experiments, 47 phenolic compounds were tentatively identified in beer including simple phenolic acids, hydroxycinnamoylquinic acids, flavanols, flavonols, flavones, alkylmethoxyphenols, alpha- and iso-alpha-acids, hydroxyphenylacetic acids and prenylflavonoids. As far as we know, 7 of these have never been determined before in beer: feruloylquinic acid, caffeic acid-*O*-hexoside, coumaric acid-*O*-hexoside, sinapic acid-*O*-hexoside, catechin-*O*-dihexoside, kaempferol-*O*-hexoside, and apigenin-*C*-hexoside-pentoside.

Phenolic compounds were identified by generating the molecular formula using accurate mass with some restrictions (C = 30, H = 100, O = 15), and matching with the isotopic pattern. This molecular formula was then identified using the Dictionary of Natural Products (Chapman & Hall/CRC). Lastly, analytes were

confirmed using MS/MS data and comparing the fragments found with the literature.

Table 1 shows the list of 47 phenolic compounds tentatively identified using LC–ESI-LTQ-Orbitrap, along with their retention time, accurate mass molecular formula and error (mDa). No differences in the phenolic profile were observed among the 4 types of beer, except for Märzenbier, which lacked catechin-*O*-hexoside.

Fig. 1 shows an FTMS chromatogram of a beer sample.

3.2. Phenolic acids

3.2.1. Hydroxybenzoic acids and derivatives

Hydroxybenzoic acids have a C_6–C_1 chemical structure and show a characteristic loss of CO_2 $[M-H-44]^-$ in MS^2 experiments (Vallverdú-Queralt, de Alvarenga, Estruch, & Lamuela-Raventos, 2013). The examination of the chromatograms in FTMS mode and the data-dependent scan revealed the presence of gallic acid (*m*/*z* 169.3014, 0.6 mDa) protocatechuic acid (*m*/*z* 153.0193, 0.06 mDa), 4-hydroxybenzoic acid (*m*/*z* 137.0241, 0.20 mDa), vanillic acid (*m*/*z* 167.0349, 0.07 mDa) and dihydroxybenzoic acid (*m*/*z* 153.0187, 0.60 mDa). All ions showed a loss of 44 u in the MS^2 spectra, and vanillic acid showed an extra fragmentation due to the loss of the methyl group $[M-H-15]^-$.

Moreover, protocatechuic acid-*O*-hexoside was also identified (*m*/*z* 315.0710, 1.10 mDa) and confirmed by MS^2 experiments, which showed the loss of the intact sugar $[M-H-162]^-$ with an error below 0.30 mDa.

3.2.2. Hydroxycinnamic acids and derivatives

Hydroxycinnamic acids have a C_6-C_3 structure with a double bond in the side chain in *cis* or *trans* configuration. Hydroxycinnamic acids are secondary metabolites generated from phenylalanine and tyrosine and are the precursors of the other polyphenol classes in plant biosynthetic pathways (El-Seedi et al., 2012). The examination of the chromatograms led to the identification of a few hydroxycinnamic acids: caffeic acid (*m*/*z* 179.0349, 0.06 mDa), *p*-coumaric acid (*m*/*z* 163.0400, 0.05 mDa), ferulic acid (*m*/*z* 193.0506, 0.06 mDa) and sinapic acid (*m*/*z* 223.0611, 0.08 mDa). The typical loss of CO_2 $[M-H-44]^-$ was observed for all hydroxycinnamic acids with an error below 0.40 mDa. Loss of a methyl group $[M-H-15]^-$ was also shown in ferulic and sinapic acid with an error of 0.1 mDa. Moreover, ferulic, *p*-coumaric and sinapic acid were confirmed comparing the retention time and the MS^2 spectrum with pure standards.

Hydroxycinnamic acid derivatives were also identified in beer samples. The MS^2 spectra showed the characteristic fragmentation involving cleavage of the sugar moiety $[M-H-162]^-$. Accurate mass measurement revealed the presence of two caffeic acid-*O*-hexosides (*m*/*z* 341.0877, 0.43 mDa), one coumaric acid-*O*-hexoside (*m*/*z* 325.0928, 0.10 mDa), one ferulic acid-*O*-hexoside (*m*/*z* 355.1034, 0.10 mDa), and two sinapic acid-*O*-hexosides (*m*/*z* 385.1139, 0.12 mDa). While caffeic acid, coumaric acid and sinapic acid have been described in beer elsewhere (Bartolomé et al., 2000; Floridi et al., 2003; Jandera et al., 2005; Montanari et al., 1999; Nardini & Ghiselli, 2004; Rehová et al., 2004), as far as we know, this is the first time that these hexoside hydroxycinnamic acid derivatives have been reported in beer.

A peak showing *m*/*z* 353.0877 revealed the presence of four caffeoylquinic acids with an error below 0.13 mDa: neochlorogenic, chlorogenic, cryptochlorogenic and 1-caffeoylquinic acids. It was possible to differentiate the four isomers by their relative intensities in MS^2 spectra according to the method cited by other authors using mass spectrometry (Clifford, Johnston, Knight, & Kuhnert, 2003; Parejo et al., 2004). All caffeoylquinic acid isomers show a characteristic fragment of *m*/*z* 191.0561 with a 0.60 mDa of error which corresponds to the quinic acid.

Also found was feruloylquinic acid (*m*/*z* 367.1034, 0.10 mDa), which is the first time it has been detected in beer, to our knowledge. MS^2 spectra of feruloylquinic acid showed the fragment of quinic acid (*m*/*z* 191.0506, 0.20 mDa) and ferulic acid (*m*/*z* 193.0506, 0.40 mDa).

3.2.3. Hydroxyphenylacetic acids

Hydrophenylacetic acids have a C_6-C_2 carbon structure and are characterised by loss of a molecule of CO_2 in MS^2 experiments. Beer is one of the major sources of hydroxyphenylacetic acids in European diets, along with olives, cider and wine (Zamora-Ros et al., 2013).

Three peaks were identified in FTMS corresponding to three hydroxyphenylacetic acid isomers (*m*/*z* 151.0395, 0.05 mDa) confirmed by the exact mass. One isomer is expected to be 4-hydroxyphenylacetic acid since this has been reported in beer previously (Nardini, Natella, Scaccini, & Ghiselli, 2006).

3.3. Flavonoids

Flavonoids are a large family of compounds with a common chemical structure: a diphenylpropane skeleton bearing two benzene rings (A and B) connected by a pyran ring attached to the A ring. Flavonoids are further divided into subclasses (anthocyanidins, chalcones, flavanols, flavanones, flavonols, flavones and isoflavonoids). Many biological effects and health benefits have been associated with flavonoid consumption, although these effects may be related to their ability to modulate cell-signalling rather than their antioxidant activity (Williams, Spencer, & Rice-Evans, 2004).

3.3.1. Flavanols and derivatives

Using LTQ-Orbitrap in FTMS and MS^2 modes, the comprehensive fragmentation pathways of (+)-catechin and (−)-epicatechin (*m*/*z* 289.0717, 0.12 mDa) was unambiguously determined. Additionally, (+)-catechin and (−)-epicatechin were confirmed with pure standards.

Two types of hexoside derivatives were found in beer samples: catechin-*O*-hexoside (*m*/*z* 451.1245, 0.13 mDa) and catechin-*O*-dihexoside (*m*/*z* 613.1773, 0.12 mDa). Both derivatives showed the loss of the sugar moiety in MS^2 spectra. Catechin-*O*-hexoside has been found in beer previously (Gerhäuser et al., 2002) but, to our knowledge, this is the first report of catechin-*O*-dihexoside in beer.

3.3.2. Flavonol derivatives

Kaempferol-3-*O*-glucoside (*m*/*z* 447.0932, 0.05 mDa) and quercetin-3-*O*-glucoside (*m*/*z* 463.088, 0.12 mDa) were found in beer by analysing the chromatograms in FTMS, for which both analytes showed the loss of 162 u due to the sugar moiety. Quercetin-3-*O*-glucoside and kaempferol-3-*O*-glucoside were further confirmed by comparing the chromatograms with pure standards. Kaempferol-3-*O*-rhamnoside, quercetin-3-*O*-rhamnoside, quercetin-3-*O*-rutinoside, quercetin-3-*O*-arabinoside and quercetin-3-*O*-glucoside have been previously reported in beer (Gerhäuser, 2005; Jandera et al., 2005; Rehová et al., 2004), but not kaempferol-3-*O*-glucoside. 3,7-dimethylquercetin (*m*/*z* 329.0666, 0.09 mDa) was also found and confirmed by MS^2 spectra showing the loss of the methyl groups $[M-H-15]^-$.

3.3.3. Flavones

The examination of the chromatograms in FTMS mode and dependent scan led to the identification of three apigenin derivatives. Apigenin-*C*-hexoside (*m*/*z* 431.0983, 0.20 mDa) showed a loss of 120 and 90 u in the MS^2 spectra characteristic of the cross-ring fissions in the sugar unit, as reported previously in

Table 1
Phenolic acids tentatively identified in beer.

Compound	Rt (min)	Accurate mass [M-H$^-$]	Fragments *m*/*z* (% intensities)	Error (mDa)	Molecular formula
Gallic acid*	1.16	169.0142	125.0241 (100)	0.60	$C_7H_6O_5$
4-Vinylguaiacol	1.46	149.0607	134.0362 (100)	0.80	$C_9H_{10}O_2$
Caffeic acid-*O*-hexoside I	1.55	341.0877	179.0345 (100), 135.0400 (10)	0.40	$C_{15}H_{18}O_9$
Caffeic acid-*O*-hexoside II	2.23	341.0877	179.0344 (100), 135.0400 (10)	0.43	$C_{15}H_{18}O_9$
Protocatechuic acid-*O*-hexoside	2.3	315.0710	153.0190 (100), 109.0291 (10)	1.10	$C_{13}H_{16}O_9$
Protocatechuic acid*	2.45	153.0193	109.0292 (100)	0.06	$C_7H_6O_4$
Catechin*	2.96	289.0717	245.0814 (100), 205.0502 (50),179.0345 (20)	0.12	$C_{15}H_{14}O_6$
Catechin-*O*-hexoside I	3.06	451.1245	289.0710, (100) 245.0817(10)	0.13	$C_{21}H_{24}O_{11}$
Neochlorogenic acid 3-caffeoylquinic acid	3.24	353.0877	191.0557 (100), 179.0349 (40), 135.0448 (10)	0.13	$C_{16}H_{18}O_9$
Hydroxyphenyl acetic acid I	3.29	151.0395	107.0499 (100)	0.05	$C_8H_8O_3$
Catechin-*O*-dihexoside	3.33	613.1773	451.1242 (100) 289.0714 (50)	0.12	$C_{27}H_{34}O_{16}$
Coumaric acid-*O*-hexoside	3.5	325.0928	163.0402 (100)	0.10	$C_{15}H_{18}O_8$
4-Hydroxybenzoic acid*	3.54	137.0241	93.0343 (100)	0.20	$C_7H_6O_3$
Hydroxyphenyl acetic acid II	3.82	151.0395	107.0498 (100)	0.05	$C_8H_8O_3$
Epicatechin*	4.19	289.0717	245.0814 (100), 205.0502 (50),179.0345 (20)	0.12	$C_{15}H_{14}O_6$
1-Caffeoylquinic acid	4.27	353.0877	191.0555 (100), 179.0343 (10)	0.11	$C_{16}H_{18}O_9$
Vanillic acid*	4.5	167.0349	152.0108 (100), 123.0447 (90), 108.0211 (30)	0.07	$C_8H_8O_4$
Chlorogenic acid 5-caffeoylquinic acid*	4.53	353.0877	191.0557 (100), 179.0346 (10), 135.0448 (1)	0.09	$C_{16}H_{18}O_9$
Catechin-*O*-hexoside II	4.56	451.1245	289.0710, (100) 245.0816 (10)	0.12	$C_{21}H_{24}O_{11}$
Caffeic acid*	4.6	179.0349	134.9875 (100)	0.06	$C_9H_8O_4$
Feruloylquinic acid	4.91	367.1034	193.0502 (100), 191.0557 (5)	0.10	$C_{17}H_{20}O_{15}$
Cryptochlorogenic acid (4-caffeoylquinic acid)	4.91	353.0877	191.0557 (100), 179.0344, 173	0.11	$C_{16}H_{18}O_9$
Hydroxyphenyl acetic acid III	5.61	151.0395	107.0498 (100)	0.05	$C_8H_8O_3$
p-Coumaric acid*	5.94	163.0400	119.0498 (100)	0.05	$C_9H_8O_3$
Sinapic acid-*O*-hexoside I	6.29	385.1139	223.0610 (100), 208.0400 (20), 179.0698 (10)	0.11	$C_{17}H_{22}O_{10}$
Ferulic acid-*O*-hexoside	6.42	355.1034	193.0507 (100), 178.0280 (10)	0.10	$C_{16}H_{20}O_9$
Indole-3-carboxylic acid	6.8	174.056	130,0657 (100)	0.06	$C_{10}H_9NO_2$
Sinapic acid-*O*-hexoside II	6.89	385.1139	223.0610 (100), 208.0403 (20), 179.0698 (10)	0.12	$C_{17}H_{22}O_{10}$
Ferulic acid*	7.03	193.0506	149.0604 (100), 178.0267 (70), 134.0370 (40)	0.06	$C_{10}H_{10}O_4$
Apigenin-C-hexoside-O-hexoside	7.39	593.1511	311.0556 (100), 431.0980 (80), 473.1086 (50), 341.0662 (20), 297.0401 (10)	0.30	$C_{27}H_{30}O_{15}$
Apigenin-C-hexoside-C-pentoside	7.93	563.1406	443.0982 (100), 473.1087 (80), 353.0664 (40), 383.0769 (30), 503.1192 (30), 545.1300(20)	0.23	$C_{26}H_{28}O_{14}$
Sinapic acid*	8.37	223.0611	208.0371 (100), 179.0708 (40), 164.0474 (20)	0.08	$C_{11}H_{12}O_5$
Apigenin-C-hexoside	9.29	431.0983	311.0551 (100), 341.0658 (40), 413.0871 (10)	0.2	$C_{21}H_{20}O_{10}$
Quercetin-3-O-glucoside*	9.57	463.0881	301.0348 (100)	0.12	$C_{21}H_{20}O_{12}$
Kaempferol-3-O-glucoside*	11.1	447.0932	285.0403 (100)	0.05	$C_{21}H_{20}O_{11}$
3,7-Dimethylquercetin	14.3	329.0666	314.0422 (100), 299.0200 (10)	0.09	$C_{17}H_{14}O_7$
Isoxanthohumol*	14.4	353.1394	233.0814 (100), 119.0499 (10)	0.07	$C_{21}H_{22}O_5$
8-Prenylnaringenin*	14.6	339.1237	219.0655 (100), 245.0811(10)	0.22	$C_{20}H_{20}O_5$
6-Prenylnaringenin	14.7	339.1237	219.0656 (100), 245.0812(10)	0.13	$C_{20}H_{20}O_5$
Cohumulone I	14.8	347.1864	235.1337 (100)	0.3	$C_{20}H_{28}O_5$
Ad-humulone	14.9	361.2020	235.1337 (100), 292.1313 (10)	1	$C_{21}H_{30}O_5$
Cohumulone II	15.3	347.1864	278.1158(100)	0.14	$C_{20}H_{28}O_5$
n-Humulone	15.5	361.2020	292.1312 (100), 343.1910 (10)	1.11	$C_{21}H_{30}O_5$
Iso-α-cohumulone	16	347.1864	251.1286(100), 329.1754(30)	0.3	$C_{20}H_{28}O_7$
Iso-α-ad/n-humulone	16.2	361.2020	265.1445 (100), 343.1916 (40), 235.1339 (10)	0.1	$C_{21}H_{30}O_7$
Iso-α-ad/n-humulone	16.4	361.2020	265.1442(100), 343.1910(40), 235.1336(10)	0.2	$C_{21}H_{30}O_8$

* Analytes confirmed by comparing with pure standards.

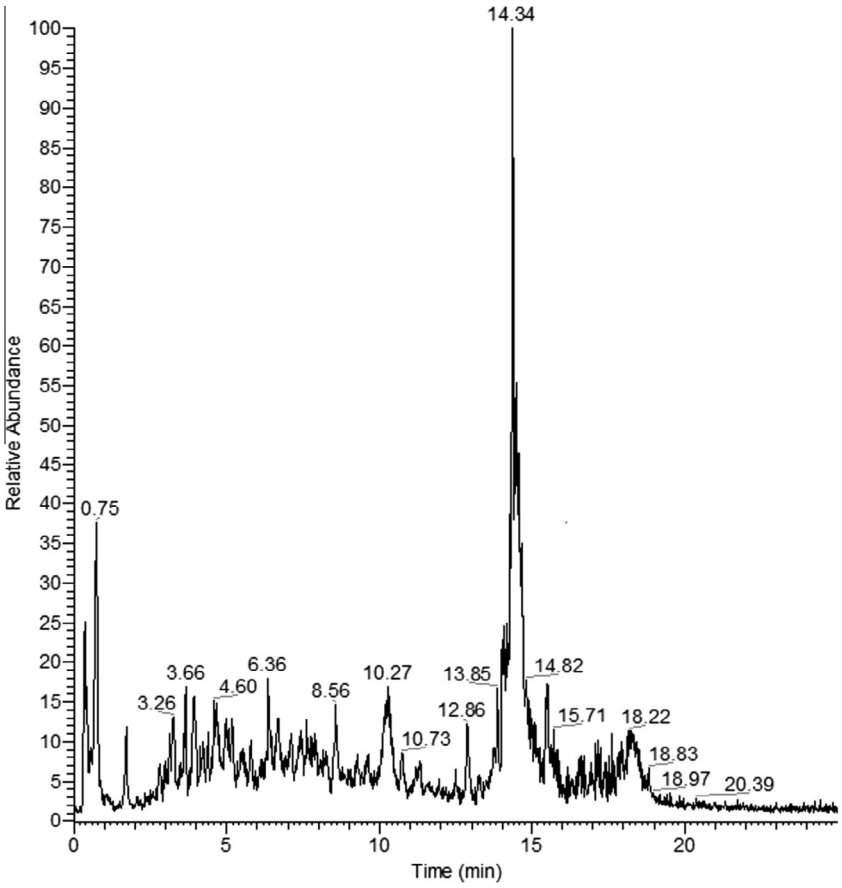

Fig. 1. Beer FTMS chromatogram.

apigenin-6,8-di-*C*-hexoside (Marín, Ferreres, Tomás-Barberán, & Gil, 2004; Parejo et al., 2004). Apigenin-*C*-hexoside-*O*-hexoside (*m*/*z* 593.1511, 0.30 mDa) was also detected and showed the two different fragmentation patterns of *C*-glycosides and *O*-glycosides in MS^2 experiments: $[M\text{-}H\text{-}120]^-$ and $[M\text{-}H\text{-}90]^-$ which corresponds to the loss of *C*-glycosides, and $[M\text{-}H\text{-}162]^-$ which agrees with the loss of the intact *O*-glycoside.

Also found was apigenin-*C*-hexoside-*C*-pentoside (*m*/*z* 563.1406, 0.23 mDa), which showed an extra loss of 90, 120 and 60 u in MS^2 spectra characteristic of cross-ring cleavage of the *C*-pentoside unit as shown in Fig. 2 and also reported previously (Marín et al., 2004; Vallverdú-Queralt, Jáuregui, Di Lecce, Andrés-Lacueva, & Lamuela-Raventós, 2011). Apigenin-*C*-glucoside and apigenin-*C*-diglucoside have been reported in beer previously (Gerhäuser et al., 2002), but as far as we know, this works demonstrates for the first time that apigenin-*C*-hexoside-*C*-pentoside is also present.

3.4. Bitter acids

Bitter acids are characteristic compounds of beer since they are synthesized in the lupulin glands of the hop plant. Bitter acids have a prenylated polyketide structure and there are two main subclasses: α-acids (humulones) and β-acids (lupulones). α-acids (n-, co-, and ad-humulone) undergo thermal isomerization during wort boiling and are transformed into iso-α-acids (isohumulone, isocohumulone and isoadhumulone) *via* an acyloin-type ring contraction (De Keukeleire, 2000). Iso-α-acids play an important role in beer organoleptic properties, since they contribute to its bitter flavour (Huvaere, Sinnaeve, Van Bocxlaer, & De Keukeleire, 2004) and the stability of the foam (Ferreira, Jorge, Nogueira, Silva, & Trugo, 2005; Kunimune & Shellhammer, 2008).

Three α-acids were identified in FTMS mode and dependent scan spectra: co-humulone (*m*/*z* 347.1864, 0.30 mDa), n-humulone and ad-humulone (*m*/*z* 361.2020, 1.11 mDa). All α-acids showed the same fragmentation pattern in MS^2 spectra: the loss of 69 u, which corresponds to the scission of the prenyl chain (3-methyl-but-2-en-1-yl, C_5H_9) with an error below 0.30 mDa. A fragment of 235.1335 (error of 0.30 mDa) was also observed in some peaks, which might be related to ring fission. Ad-humulone and n-humulone were distinguished according to the intensity of the *m*/*z* 292.1312 fragment, as reported previously (Hofte & Van Der Hoeven, 1998), the peak with a greater intensity of the mass fragment being matched to n-humulone. A co-humulone isomer was also detected, although the two compounds could not be discriminated based on their mass fragment intensities.

Three iso-α-acids were also found in beer: iso-α-cohumulone, iso-α-adhumulone and iso-α-humulone. Although iso-α-acids have the same molecular weight as α-acids, they could be distinguished by the different fragmentation pattern. Iso-α-acids showed a characteristic loss of 96 u, which corresponds to the scission of the side chain (4-methyl-pent-3-en-1-oxo, C_6H_8O) instead of 69 u (C_5H_9). Iso-α-acids were identified with an error below 0.40 mDa. Iso-α-adhumulone and iso-α-humulone could not be distinguished since both peaks had the same fragmentation pattern and intensities.

3.5. Prenylflavonoids

Prenylflavonoids are a sub-class of flavonoids, mainly found in hops, characterised by having a prenyl group attached in the flavanoid backbone (ring A). Female inflorescences of hops used in brewing are particularly rich in xanthohumol, although during the brewing process, xanthohumol isomerizes into

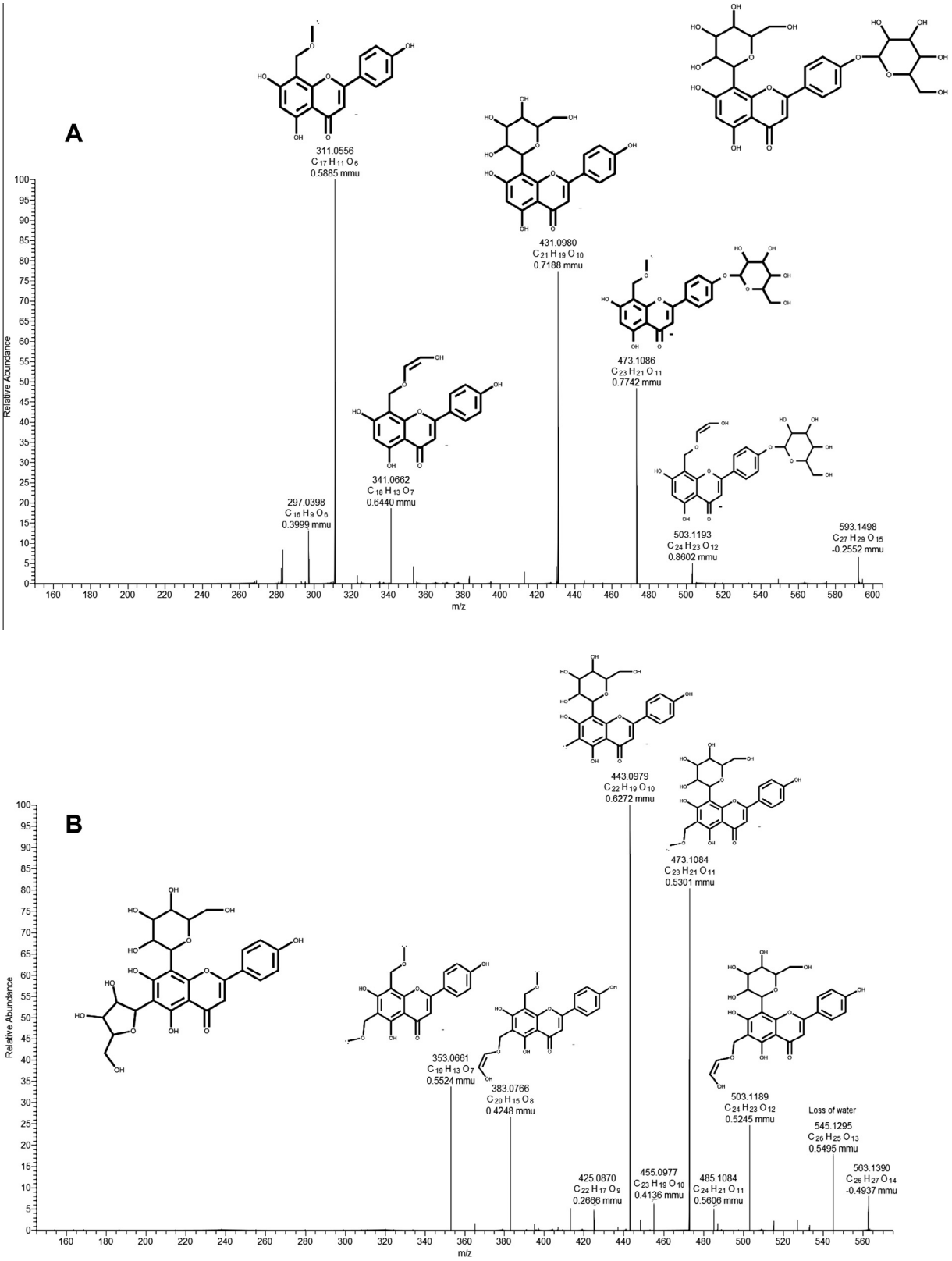

Fig. 2. MS^2 spectra of apigenin-*C*-hexoside-*O*-hexoside (A) and apigenin-*C*-hexoside-*C*-pentoside (B).

isoxanthohumol (Stevens, Taylor, Clawson, & Deinzer, 1999). Other important hop flavanoids previously found in beer are desmethylxanthohumol, 6-prenylnaringenin 8-prenylnarigenin and 6-geranylnaringenin (Stevens, Taylor, & Deinzer, 1999).

All prenylflavonoids follow the same fragmentation pattern and show ions corresponding to retro-Diels Alder fragmentation in the C-ring, involving 1,3 scission in MS^2 spectra, as described previously in other flavonoids like naringenin (Vallverdu-Queralt,

Jauregui, Medina-Remon, Andres-Lacueva, & Lamuela-Raventos, 2010).

Isoxanthohumol (*m*/*z* 353.1394, 0.07 mDa), 8-prenylnaringenin and 6-prenylnaringenin (*m*/*z* 339.1237, 0.22 mDa and 0.13 mDa, respectively) were found in beer samples and confirmed using MS^2 data. Isoxanthohumol and 8-prenylnaringenin were further confirmed by comparison with pure standards.

3.6. Alkylmethoxyphenols

Flavour-active volatile phenols, such as vanillin, acetovanillone, 4-vinylsyringol, 4-vinylguaiacol and 4-vinylphenol, have been described in beer previously (Vanbeneden, Gils, Delvaux, & Delvaux, 2008). In particular, ferulic acid releases 4-vinylguaicol through decarboxylation by thermal decomposition during the wort boiling (McMurrough et al., 1996) and by enzymatic reaction during fermentation and brewing (Coghe, Benoot, Delvaux, Vanderhaegen, & Delvaux, 2004).

The higher sensitivity of the LTQ-Orbitrap system enabled the identification of low-intensity signals, such as 4-vinylguaicol (*m*/*z* 149.0607, 0.80 mDa). MS^2 spectrum confirmed the presence of this compound, showing a loss of the methyl group $[M\text{-}H\text{-}15]^-$.

3.7. Indole-based compounds

Indole-3-carboxylic acid (*m*/*z* 174.0560, 0.06 mDa) was also found in beer samples. MS^2 spectra showed a loss of CO_2 $[M\text{-}H\text{-}44]^-$. Indole-3-carboxylic acid is a product of the biotransformation of the indole alkaloid gramine, which is found in barley (Digenis, 1969).

4. Conclusion

Using an LTQ-Orbitrap high resolution mass spectrometer, we were able to identify 47 phenolic compounds in beer, seven of which, as far as we know, are reported for the first time. Most of these polyphenols are hexosides, dihexosides, pentosides and quinic conjugates, such as feruloylquinic acid, caffeic acid-*O*-hexoside, coumaric acid-*O*-hexoside, sinapic acid-*O*- hexoside, catechin-*O*-dihexoside, kaempferol-*O*-hexoside, and apigenin-*C*-hexoside-pentoside. LC–ESI-LTQ-Orbitrap-MS allowed the characterisation of the polyphenols present in beer, based on the accurate mass measurement with a low error (<1.1 mDa) and the MS^2 spectra data. The phenolic compounds were further confirmed by comparisons with pure standards whenever possible as well as with the literature.

This study broadens knowledge of beer polyphenols, which might be helpful for further research on the health and sensory properties of beer.

Author disclosures

Dr. Estruch reports serving on the board of and receiving lecture fees from the Research Foundation on Wine and Nutrition (FIVIN); serving on the boards of the Beer and Health Foundation and the European Foundation for Alcohol Research (ERAB); receiving lecture fees from Cerveceros de España and Sanofi-Aventis; and receiving grant support through his institution from Novartis. Dr. Lamuela-Raventos reports serving on the board of and receiving lecture fees from FIVIN; receiving lecture fees from Cerveceros de España; and receiving lecture fees and travel support from PepsiCo. Nevertheless, these foundations had no involvement in the study design, the collection, analysis and interpretation of data, the writing of the manuscript or the decision to submit the manuscript for publication. The other authors declare no conflict of interest.

Acknowledgements

This study was supported by The European Foundation for Alcohol Research (ERAB) EA 1117 and EA 1324 and in part by CICYT (AGL2010-22319-C03; AGL2013-49083-C3-1-R), the Instituto de Salud Carlos III, ISCIII (CIBERobn) from the Spanish Ministry of Science and Innovation Ministerio de Ciencia e Innovación (MICINN) and Generalitat de Catalunya (GC) 2014 SGR 773. Paola Quifer-Rada is grateful for the predoctoral fellowships awarded by the Generalitat de Catalunya (FI-DRG). Miriam Martínez-Huélamo thanks the predoctoral programme of MICINN.

All authors have read and approved the final manuscript.

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4.2. Biomarkers of beer consumption. Validation of Isoxanthohumol as an accurate biomarker, discovery of potential new biomarkers of beer consumption and metabolomics

4.2.1. Publication 2. Analytical condition setting a crucial step in the quantification of unstable polyphenols in acidic conditions: analyzing prenylflavanoids in biological samples by liquid chromatography-electrospray ionization triple quadruple mass spectrometry.

Paola Quifer-Rada, Miriam Martínez-Huélamo, Olga Jáuregui, Gemma Chiva-Blanch, Ramón Estruch, and Rosa M. Lamuela-Raventós. *Analytical Chemistry*, 2013 4;85(11):5547-54.

Summary:

The interest in studying hops and beer prenylflavanoids, isoxanthohumol, xanthohumol and 8-prenylnaringenin, has increased in recent years due to their biological activity as strong phytoestrogens and potent cancer chemopreventive agents. However, prenylflavanoids behave differently from most polyphenols, since they are unstable at acidic pH. To our knowledge, no published studies to date have considered the degradation of these compounds during analytical processes. In the present work, a new sensitive and specific method based on solid phase extraction and liquid chromatography coupled to electrospray ionization triple quadruple mass spectrometry (LC-ESI-MS/MS) was developed and validated. The new method was optimized to avoid degradation of the selected analytes, isoxanthohumol, xanthohumol and 8-prenylnaringenin, throughout the analytical process and to reduce the urine matrix effect in LC-ESI-MS/MS assays. In order to achieve this goal, different analytical parameters where studied including chromatographic mobile phases combination and pH, selection of the most suitable SPE cartridge, pH used during SPE and optimization of the clean-up step of urine.

It was concluded that a neutral pH (pH 7.0) is necessary for the analysis of prenylflavanoids, in order to maintain the stability of compounds for at least 24 hours. The addition of ascorbic acid to the media improved stability, calibration curves coefficients of correlation, accuracy, and precision parameters. Mix-mode cation exchange sorbent yielded the best matrix effect factors and recoveries. Method validation results showed appropriate intra-day and inter-day accuracy and precision (<15%). Recovery of isoxanthohumol, xanthohumol, and 8-prenylnaringenin was 97.1%±0.03, 105.8%±0.05 and 105.4%±0.04, respectively, and matrix effect factors were nearly 100%. The stability assay showed that analytes were stable for at least 24 hours. Moreover, the

method was applied to quantify 10 human samples of urine and was able to quantify prenylflavanoids in urine after the consumption of a single dose of beer (330 mL).

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Analytical Condition Setting a Crucial Step in the Quantification of Unstable Polyphenols in Acidic Conditions: Analyzing Prenylflavanoids in Biological Samples by Liquid Chromatography–Electrospray Ionization Triple Quadruple Mass Spectrometry

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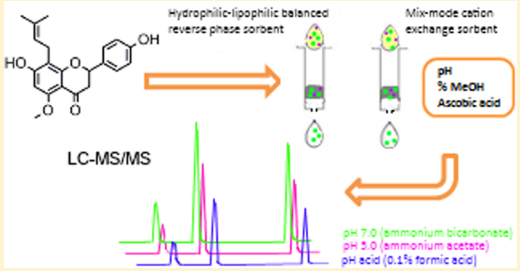
ABSTRACT: The interest in studying hops and beer prenylflavanoids, isoxanthohumol, xanthohumol, and 8-prenylnaringenin, has increased in recent years due to their biological activity as strong phytoestrogens and potent cancer chemopreventive agents. However, prenylflavanoids behave differently from most polyphenols, since they are unstable at acidic pH. To our knowledge, no published studies to date have considered the degradation of these compounds during analytical processes. In the present work, a new sensitive and specific method based on solid phase extraction and liquid chromatography coupled to electrospray ionization triple quadruple mass spectrometry (LC–ESI-MS/MS) was developed and validated. The new method was optimized to avoid degradation of the selected analytes, isoxanthohumol, xanthohumol, and 8-prenylnaringenin, throughout the analytical process and to reduce the urine matrix effect in LC–ESI-MS/MS assays. It was concluded that a neutral pH (pH 7.0) is necessary for the analysis of prenylflavanoids, in order to maintain the stability of compounds for at least 24 h. The addition of ascorbic acid to the media improved stability, calibration curves, coefficients of correlation, accuracy, and precision parameters. Mix-mode cation exchange sorbent yielded the best matrix effect factors and recoveries. Method validation results showed appropriate intraday and interday accuracy and precision (<15%). Recovery of isoxanthohumol, xanthohumol, and 8-prenylnaringenin was 97.1% ± 0.03, 105.8% ± 0.05, and 105.4% ± 0.04, respectively, and matrix effect factors were nearly 100%. The stability assay showed that analytes were stable for at least 24 h. The method was applied to quantify 10 human samples of urine and was able to quantify prenylflavanoids in urine after the consumption of a single dose of beer (330 mL).



Prenylflavanoids are mainly found in hops (*Humulus lupulus L.* cannabaceae) and products made with hops, such as beer and herbal extracts. The interest in studying prenylflavanoids has increased due to their reported biological activity. 8-Prenylnaringenin (8PN) is the strongest phytoestrogen identified to date,[1,2] presenting an ERα agonist activity 100 times more potent than genistein, the well-recognized soy phytoestrogen.[2] Owing to this phytoestrogenic activity, 8PN has been proposed as a treatment for menopausal symptoms, such as hot flashes and osteoporosis. Xanthohumol (XN) and isoxanthohumol (IX) have shown weak or no estrogenic activity.[3] However, IX is considered to be a source of 8PN, because it is metabolized to 8PN in the intestinal tract by an *O*-demethylation catalyzed by gut microbiota.[4,5]On the other hand, XN has been described as a potent cancer chemopreventive agent[6−8] and has shown antiproliferative activity in cancer cell lines of the human breast (MCF-7), colon (HT-29), and ovary (A-2780).[7]

The main problem in the analysis and quantification of these compounds is that prenylflavanoids behave differently from other phenolic compounds. In an acidic medium, cyclization of the prenyl side chain[9] gives IX, 8PN, and XN a four-ring structure (Figure 1), while under basic conditions XN is converted into IX by the Michael addition reaction.[10] These chemical features lead to a low reproducibility in the quantification of these compounds in biological samples. To our knowledge, specific and fully validated methods to analyze IX, XN, and 8PN by LC–MS/MS in biological samples are not found in the literature. Thereby, there is a need for a robust

Received: March 14, 2013
Accepted: May 3, 2013
Published: May 3, 2013

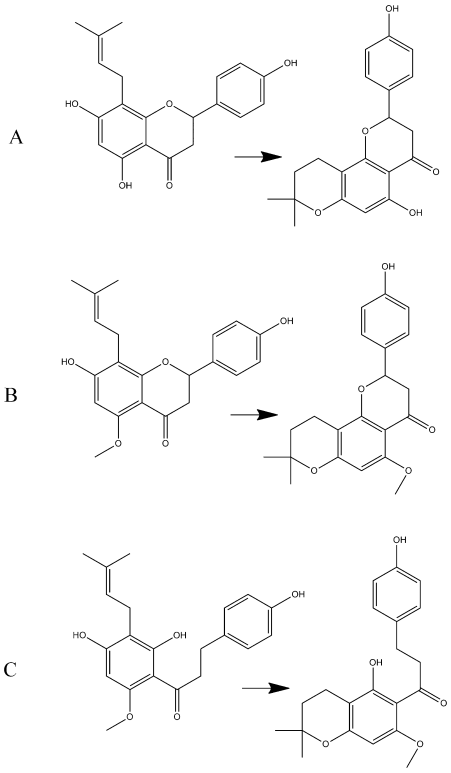


Figure 1. Cyclization of the prenyl side chain of 8-prenylnaringenin (A), isoxanthohumol (B), and xanthohumol (C) in acidic medium.

analytical method to identify and quantify IX, XN, and 8PN in biological matrices such as urine.

In the present work, a new specific method was developed and validated to identify and quantify IX, XN, and 8PN in urine samples by LC−MS/MS. This method was optimized to maintain analyte stability throughout the analytical process. A solid phase extraction (SPE) study was performed comparing two different sorbents to optimize the procedure and reduce the matrix effect of urine. Moreover, variables that could affect analytes quantification and stability were explored, such as pH during the SPE procedure, pH during the HPLC analysis, the use of ascorbic acid (AA) as antioxidant, and the effect of the mobile phase used in the chromatographic separation. Finally, human urine from young male volunteers who drank a moderate dose of 330 mL of beer was analyzed to demonstrate the applicability of the method.

EXPERIMENTAL SECTION

Reagents and Materials. Isoxanthohumol, 8-prenylnaringenin, and xanthohumol (97−99% purity) were purchased from Enzo Life Science (Lausen, Switzerland). Taxifolin (>90% purity) was obtained from Extrasynthèse (Genay, France). Ascorbic acid, methanol (MeOH), acetonitrile (MeCN) and ammonium bicarbonate of HPLC grade were supplied by Sigma-Aldrich (St. Louis, MO). Acetic acid, formic acid, and ammonium acetate of HPLC grade were purchased from Panreac Quimica SA (Barcelona, Spain). Ultrapure water (Milli-Q) was generated by a Millipore System (Bedford). SPE cartridges, Oasis HLB 96-well plates 30 μm (30 mg) and Oasis MCX 96-well plates 60 μm (30 mg) were obtained from Waters (Milford, MA).

SPE Extraction Method. Oasis MCX 96-well plates were equilibrated with 1 mL of MeOH and 1 mL of 5 mM ammonium acetate buffer adjusted to pH 5.0. Then 1 mL of previously centrifuged urine was loaded into the cartridge. Samples were washed with 1 mL of MeOH/5 mM aqueous ammonium acetate buffer at pH 5 (1:1, v/v) solution and eluted with 0.5 mL of MeOH (×2). The eluate obtained was evaporated to dryness under a gentle stream of N_2, and the residue was reconstituted with 100 μL of 5 mM ammonium bicarbonate buffer at pH 7 containing 20 mg L^{-1} of ascorbic acid.

LC−ESI-MS/MS Equipment. LC analyses were performed using an HP Agilent 1100 system equipped with a quaternary pump and a refrigerated autosampler (Waldbronn, Germany) coupled to an API 3000 triple-quadruple mass spectrometer (AB Sciex, Framinghan, MA) with a turbo ion spray source working in negative mode. Chromatographic separation was performed with a Phenomenex Luna C_{18} column, 50 mm × 2.0 mm i.d, 5 μm (Torrance, CA) and coupled to a precolumn Phenomenex security guard C_{18} (4 × 2 mm i.d.). The system and acquisition data were controlled by Analyst v 1.4.2 software supplied by AB Sciex (Framinghan, MA).

Chromatographic Conditions. Gradient elution of analytes was carried out with 5 mM ammonium bicarbonate buffer adjusted to pH 7.0 as the aqueous mobile phase (A) and acetonitrile and methanol as organic phases in a proportion of 1:1. A nonlinear gradient was applied: 0 min, 95% A; 0−1 min, 87% A; 1−2.5 min, 60% A; 2.5−5 min, 30% A; 5−7 min, 0% A; 7−8 min, 0% A; 8−8.5 min, 95% A; 8.5−11 min, 95% A. To improve chromatographic reproducibility, the column was maintained at 40 °C. The injection volume was 20 μL, and the flow rate was 600 μL min^{-1}.

MS/MS Parameters. Multiple reaction monitoring (MRM) mode was used to identify and quantify analytes. MS/MS parameters for working in MRM mode were optimized by direct infusion of each individual standard at a concentration of 1 mg L^{-1} in 50:50 (v/v) 5 mM ammonium bicarbonate buffer at pH 7/MeOH/MeCN (1:1) into the mass spectrometer using a syringe pump (Harvard Apparatus, Holliston, MA) at a constant flow rate of 10 μL min^{-1}. The turbo ion spray source was used in negative mode with the following settings: capillary voltage −4000 V, nebulizer gas (N_2) 10 (arbitrary units), curtain gas (N_2) 12 (arbitrary units), and drying gas (N_2) heated to 400 °C and introduced at a flow-rate of 6000 mL min^{-1}. The ions in MRM mode were produced by collision-

Table 1. MS/MS Parameters Optimized and Transitions Used to Identify and Quantify in MRM[a]

	MRM transitions	DP (V)	FP (V)	CE (V)	EP (V)	dwell time (ms)
taxifolin	303 → 285	−50	−200	−20	−10	1000
isoxanthohumol	353 → 119	−60	−200	−35	−11	600
8-prenylnaringenin	339 → 219	−60	−210	−30	−11	600
xanthohumol	353 → 119	−60	−200	−35	−11	600

[a]DP, declustering potential; FP, focusing potential, EP, entrance potential; CE, collision energy.

dx.doi.org/10.1021/ac4007733 | Anal. Chem. 2013, 85, 5547−5554

activated dissociation (CAD) of selected precursor ions in the collision cell of the triple quadruple and analyzed with the second quadruple of the instrument. The optimum CAD (N_2) was 4 (arbitrary units).

Table 1 shows MRM transitions, declustering potential (DP), focusing potential (FP), collision energy (CE), entrance potential (EP), and dwell time for each analyte.

Method Validation. The method was fully validated following the guidance for industry of the U.S. Food and Drug Administration (FDA) for the Validation of Bioanalytical Methods.[11] The parameters evaluated were inter- and intraday accuracy and precision, recovery, limits of detection (LOD) and quantification (LOQ), selectivity, linearity, and stability. Because of the instability of the analytes observed in our work and described previously,[9] two stability assays were carried out: postpreparative stability and freeze and thaw stability.

Postpreparative stability, which evaluates the stability of processed samples, including the resident time in the autosampler refrigerated at 4 °C, was determined by preparing 2 concentrations (50 $\mu g\ L^{-1}$ and 150 $\mu g\ L^{-1}$) in blank urine in triplicate. Urine was extracted as described in SPE Extraction Method and injected into the LC−MS/MS system at the initial time for quantification. After 24 h, samples were reinjected, quantified, and compared with the initial value. A time of 24 h was chosen as the estimated waiting time of a whole 96-well plate in the autosampler, taking into account chromatographic analysis time and the number of samples.

Freeze and thaw stability was assessed by spiking blank urine at 2 concentrations (5 $\mu g\ L^{-1}$ and 30 $\mu g\ L^{-1}$) in triplicate and extracting it as described in SPE Extraction Method. Urine was injected into the LC−MS/MS system and quantified using a calibration curve prepared simultaneously. Urine was then frozen at −80 °C and thawed at ambient temperature after 24 h. The freeze and thaw cycle was performed three times, and at the third cycle, urine was reinjected into the LC−MS/MS system, quantified and compared with the initial concentration.

In addition, the matrix effect was evaluated using the method suggested by Matuszewski et al.,[12] which employs the following equation: matrix effect factors are calculated as the ratio (expressed in percentage) of the peak area of the analytes recorded for the sample spiked with the standards after SPE extraction and the peak area of analytes recorded for the standard solution. Therefore, when the sample matrix exerts a suppressive effect, the matrix factor is lower than 100%. In contrast, a matrix effect factor higher than 100% indicates signal enhancement.

Calibration Standards. Individual stock solutions of IX, XN, and 8PN were prepared at a concentration of 1 $mg\ mL^{-1}$ in methanol. The working solutions used to spike urine samples were prepared by mixing the individual standard solutions and diluting to known concentrations with 5 mM ammonium acetate buffer adjusted to pH 5.0. A taxifolin solution at a concentration of 600 $\mu g\ L^{-1}$ was used as the internal standard. Calibration curves were prepared by spiking urine in duplicate at known concentrations ranging from 0 to 40 $\mu g\ L^{-1}$ of standards and internal standard (IS) (600 $\mu g\ L^{-1}$). Urine samples were then extracted by SPE as described in SPE Extraction Method. Peak area ratios of analytes and the internal standard were plotted against the nominal concentration of the standards. Weighted linear regression analysis was used. Weighting was applied to select the best curve fit that provided residual plots lower than 15%.

Linearity was assessed in the range of 3 and 1000 $\mu g\ L^{-1}$. The adequacy of the model and linearity was evaluated by correlation coefficients (r^2). To ensure acceptable accuracy of the calibration curve, relative residual plots did not exceed 15%. The LOD and LOQ were estimated as the concentration of analytes with a signal-to-noise ratio of 3 and 10, respectively, as long as the accuracy and precision values did not exceed 20%.

Buffer and Solution Preparation. In the SPE and LC method development, buffers were assayed at different pH. Thereby, formic acid solutions were set at the desired pH (pH 2.1, 2.7, 3.0, and 4.0). A 5 mM ammonium acetate buffer was used to prepare a solution of pH 6.7, which was then adjusted to pH 5.0. pH 7.0 buffer was prepared using 5 mM ammonium bicarbonate adjusted with acetic acid.

Study Design and Samples. A total of 10 healthy young men (21−39 years old) drank a moderate dose (330 mL) of a previously selected beer (pale lager beer) with known content of IX, XN, and 8PN (see Analysis of Beer Samples section). After 8 h (in the morning), a spot urine sample was collected. Before the intervention, a 4-day washout period was set, during which volunteers were asked not to consume products based on hops, such as beer, nonalcoholic beer, or hop extract. After the washout period, a blank spot urine sample was also collected in the morning. Volunteers who reported a prior medical history of a serious illness, alcoholism, or other drug addiction or had been under medication were excluded from the study. Urine samples were collected in sterilized sample pots, aliquoted and immediately frozen at −80 °C until analysis.

The study protocol was approved by the Ethics Committee of the University of Barcelona (Institutional Review Board IRB00003099). The volunteers were fully informed and gave their written consent.

Creatinine Determination. Creatinine analysis was performed using the Jaffé alkaline picrate method[13] adapted to microtiter 96-well plates.[14] Briefly, 3 μL of urine was mixed with 60 μL of aqueous picric acid solution (1%) and 5 μL of sodium hydroxide (10%). Then, after 15 min in darkness at room temperature, 232 μL of Milli-Q water was added. The absorbance was measured at 500 nm by a UV−vis spectrophotometer. To quantify creatinine content in urine samples, calibration curves were prepared with a creatinine standard.

Statistical Analysis. Statistical analysis was performed using IBM SPSS Statistics version 19. The Mann−Whitney test for unrelated samples was performed to compare changes in prenylflavanoids urinary excretion after the washout period and intervention. Statistical tests were two-tailed and the significance level was considered as $P < 0.05$.

Safety Considerations. General guidelines for working with organic solvents and acids were followed. Universal precautions for handling biological samples and chemicals were taken.

RESULTS AND DISCUSSION

Method Development. *LC Method. Organic Mobile Phases.* Although binary mobile phases are most commonly used in reversed phase liquid chromatography, ternary mobile phases may be employed in order to exploit their unique chromatographic selectivity.[15,16] In this work, a ternary mobile phase consisting of 5 mM ammonium bicarbonate adjusted to pH 7.0, methanol and acetonitrile was used to improve peak shape and decrease background noise compared to the binary mobile phase. Tailing peaks were obtained when using only

dx.doi.org/10.1021/ac4007733 | Anal. Chem. 2013, 85, 5547−5554

Table 2. Coefficient Correlation, Accuracy, Precision, and Stability Obtained in Different Calibration Curve Media

				accuracy (%)			precision (% CV)			stability (% of degradation)					
	r^2			n = 10			n = 10			12 h			24 h		
medium	IX	8PN	XN	IX	8PN	XN	IX	8PN	XN	IX	8PN	XN	IX	8PN	XN
pH 2.7	0.82	0.84	0.60	1.6	4.4	>15	10.6	13	>15	–[a]	–[a]	–[a]	–[a]	–[a]	–[a]
pH 4	0.98	0.99	0.94	5	5	>15	8.8	8.3	>15	–[a]	–[a]	–[a]	–[a]	–[a]	–[a]
Milli-Q water	0.94	0.97	0.97	11	3.5	>15	12.4	5.2	>15	–[a]	–[a]	–[a]	–[a]	–[a]	–[a]
pH 5	0.97	0.97	0.97	14	1	>15	7.1	3.8	>15	7.9	37	91	–[a]	–[a]	–[a]
pH 5 + AA[b]	1.00	1.00	0.97	7.8	6.8	>15	4.2	3.8	2.5	0	13.5	12.2	4.5	33.3	32.2
pH 7 + AA	1.00	0.98	1.00	11	12	15	2.2	13.2	3.5	0	0	7.8	0	3.72	15.7

[a]Stability not determined since results in accuracy and precision obtained were not acceptable. [b]AA, ascorbic acid.

MeOH as the organic mobile phase. In contrast, MeCN provided symmetrical peaks but increased background noise. The combination of MeCN and MeOH in a proportion of 1:1 produced reproducible results, good peak resolution, and low background noise.

Choice of Reconstitution Media Conditions and Aqueous Mobile Phase. A pH study of the reconstitution medium after SPE and the aqueous mobile phase was performed to reduce the aforementioned analyte instability[9] observed in our analysis. This assay was one of the most critical steps in obtaining a reproducible and validated method.

We first studied the influence of the pH medium on the stability of prenylflavanoids. For this purpose, 5 mM ammonium acetate buffer adjusted to pH 5.0 was chosen as the aqueous mobile phase. Duplicated six-point calibration curves ranging from 0 to 200 μg L^{-1} were prepared using media of pH 2.7, 4.0, 5.0, and 5.8 (Milli-Q water). Taxifolin was used as the internal standard at 600 μg L^{-1}. A midpoint of all calibration curves (150 μg L^{-1}) was injected 10 times to assess accuracy and repeatability.

The reconstitution medium of pH 5.0 showed the best coefficients of correlation, accuracy, and precision parameters (Table 2). However, when these calibration curves were reinjected after 12 h, a significant loss of analyte signal was observed (decrease of 7.9%, 37%, and 91% for IX, 8PN, and XN, respectively), probably due to degradation and/or oxidation during the waiting time in the LC autosampler at 4 °C. Thus, in order to avoid analyte oxidation, new calibration curves at pH 5.0 were prepared adding ascorbic acid (20 mg L^{-1}). After injecting the calibration curves every 12 h for 3 days, at 12 h analyte stability was found to have improved due to the antioxidant activity of ascorbic acid but still needed further improvement, since a reduced signal for IX, XN, and 8PN of 4.5%, 32%, and 33%, respectively, was observed after 24 h.

Therefore, the pH of the medium and mobile phase was increased to 7.0 using 5 mM ammonium bicarbonate buffer and 20 mg L^{-1} of ascorbic acid was also added. pH 7.0 did not affect analyte ionization, since compound pK_a ranged from 7 to 8 (pK_{aXN} = 7.5, pK_{aIX} = 7.9, pK_{a8PN} = 7.7). The calibration curve was injected at the beginning (t = 0) and at 12 and 24 h, and a midpoint (150 μg L^{-1}) was injected 10 times to assess accuracy and repeatability.

When the medium pH was increased, the calibration curves showed higher coefficient correlation (r^2>0.99), accuracy, and precision values (<15%) (Table 2). This effect was enhanced when ascorbic acid was added to the medium (Table 2). Moreover, under these conditions, analytes remained stable after 24 h, especially IX and 8PN (Table 2).

Thus, to ensure prenylflavanoids stability, 5 mM ammonium bicarbonate buffer adjusted to pH 7.0 containing 20 mg L^{-1} of ascorbic acid was chosen as the reconstitution medium. Also, the aqueous mobile phase was set at pH 7.0 using ammonium bicarbonate to maintain the same pH throughout the chromatographic process.

SPE Method. SPE Cartridge Selection. Two different chemical cartridges were tested for the extraction of XN, IX, and 8PN: Oasis HLB (hydrophilic–lipophilic-balance cartridge) and Oasis MCX (mixed-mode cation-exchange and reversed-phase solvent). Blank urine was spiked with 2000 μg L^{-1} of XN, IX, and 8PN and 400 μg L^{-1} of internal standard and was loaded into a preconditioned SPE cartridge for sample cleanup and concentration.

Oasis HLB SPE procedure was performed using the method previously described by Martínez-Huélamo et al.[17] for the analysis of polyphenols and their metabolites in urine samples. Briefly, cartridges were activated with MeOH and 5.5% formic acid (v/v). Samples were washed with 5.5% formic acid (v/v) and then 5% MeOH (v/v) solution. Analytes were eluted with MeOH acidified with 0.1% formic acid (v/v). The eluate was evaporated to dryness under a gentle stream of N_2. The residue was reconstituted with water acidified with 0.1% formic acid (v/v).

The Waters Oasis MCX generic protocol was applied to extract analytes from the urine matrix. The sorbent was conditioned with MeOH and 2% formic acid (v/v) solution. Then samples were loaded and washed with 2% formic acid (v/v) solution. Analytes were eluted with MeOH, and the eluate obtained was evaporated to dryness under a gentle stream of N_2. The residue was reconstituted with water acidified with 0.1% formic acid (v/v).

In order to assess extraction recovery, blank urine extracts spiked after SPE were also prepared at the same concentration. Additionally, pure standards in mobile phase were injected to evaluate the matrix effect using the method published by Matuszewski et al.[12]

Using the Oasis HLB and MCX original protocols explained above, signal suppression of analytes was too high (94–96%), so the percentage of MeOH in the cleanup step was increased up to 40% in both extractions.

Table 3 compares the recoveries and matrix effect factors in urine obtained with Oasis HLB and MCX. The best recoveries were obtained with Oasis MCX, which also removed more urine matrix interferences, providing higher matrix effect factors than those of Oasis HLB (70%, 58%, and 15% for IX, 8PN, and XN, respectively). For these reasons, Oasis MCX was chosen as the sorbent for SPE, although a more exhaustive optimization of the extraction protocol was still required to perform a more

dx.doi.org/10.1021/ac4007733 | Anal. Chem. 2013, 85, 5547–5554

Table 3. Recovery and Matrix Effect Obtained in Oasis HLB and Oasis MCX Sorbents[a]

	recovery (%)			matrix effect (%)		
sorbent	IX	8PN	XN	IX	8 PN	XN
HLB	94.6	82.7	n.d.	14.8	6.17	n.d
MCX	115	110.1	62.4	70.8	58.1	15.0

[a]n.d.: not detected.

thorough cleanup with improved matrix effect factors, especially for XN and 8PN (see Choice of the Percentage of MeOH Applied in the Oasis MCX Cleanup Step).

Choice of the Percentage of MeOH Applied in the Oasis MCX Cleanup Step. In order to remove interferences from the urine samples, different amounts of MeOH(v/v) in water (5%, 10%, 15%, 20%, 30%, 40%, 50%, and 60%) were applied in the cleanup step. In this assay, to evaluate analyte recoveries, blank urine was spiked before and after extraction with 5000 $\mu g\ L^{-1}$ of the standards and 400 $\mu g\ L^{-1}$ of internal standard in triplicate. LOD were calculated to evaluate signal suppression caused by the matrix; the lower the LOD, the less the signal suppression.

LOD drastically improved when applying 30% of MeOH (v/v) in the cleanup step (3 $\mu g\ L^{-1}$, 1.7 $\mu g\ L^{-1}$, and 4.5 $\mu g\ L^{-1}$ for IX, 8PN, and XN, respectively) compared to the LODs compared to lower percentages of MeOH (>11 $\mu g\ L^{-1}$, >6.1 $\mu g\ L^{-1}$, and >17 $\mu g\ L^{-1}$ for IX, 8PN, and XN, respectively).

The best recoveries were obtained when applying 50% MeOH (v/v) in the cleanup step, since it removed interferences and allowed optimum analyte detection. Using <50% MeOH may lead to an underestimation of recovery, which is strongly influenced by the matrix effect since they have a tight impact on each other thus. High matrix effect could incorrectly estimate recovery (underestimate or overestimate depending on the matrix) as well as process efficiency.[18]

The best LOD were obtained when cleaning with 60% MeOH (v/v), although under these conditions the internal standard was eluted and calculation of analyte recovery was impossible. Hence, 50% MeOH concentration was chosen for the cleanup in the Oasis MCX protocol to remove urine interferences and decrease ion suppression.

Choice of Suitable pH for Extraction with MCX Cartridges. Prenylflavanoids undergo degradation in acidic media, acquiring a four-ring structure through cyclization of the prenyl chain[9] (Figure 1). To avoid degradation of IX, XN, and 8PN during the Oasis MCX extraction, the pH of the original protocol was increased. A ramp of different pHs was applied in the extraction procedure: pH 2.1, 3.0, 4.0, 5.0, and 6.7. In the cleanup step, 50% MeOH (v/v) solution was applied. Blank urine was spiked before and after extraction at three different concentrations (15, 60, and 200 $\mu g\ L^{-1}$) to calculate analyte recoveries. Also, LOD were calculated for each pH during the extraction to ensure that signal suppression remained low, independent of the pH applied.

The results showed that analyte recoveries were influenced by the pH applied during the extraction. When pH was lower than 5.0, recoveries were low (<90%), improving at pH 5.0 (>90%). Yet when pH was higher than 5.0, analyte recoveries declined, suggesting that the cation exchange interaction of the sorbent was broken and urine interferences were eluted, increasing the matrix effect and leading to underestimated recoveries.[18] Hence, the Oasis MCX extraction was established with a 5 mM ammonium acetate buffer at pH 5.0 as the elution solvent.

Method Validation. *Accuracy and Precision.* Intra- and interday accuracy and precision were evaluated by spiking blank urine with three known concentrations: low (5 $\mu g\ L^{-1}$), medium (15 $\mu g\ L^{-1}$), and high (30 $\mu g\ L^{-1}$) in five replicates. The experiment was repeated on three different days (n = 15). Accuracy was calculated as the ratio of the mean observed concentration and the known spiked concentration in the blank urine matrix. Precision is expressed as the coefficient of variation of all determinations.

According to the guidance for industry of the U.S. Food and Drug Administration (FDA) for the Validation of Bioanalytical Methods, accuracy and precision values should be within 15%, except at LOQ, where it should not exceed 20%.[11] The intraday and interday accuracy and precision of analytes in the urine matrix met the acceptance criteria of the FDA at each concentration (<15%). Accuracy in urine ranged from 1 to 14.6% and precision from 2.8 to 13.7% (Table 4).

Recovery and Matrix Effect. Recovery was assessed by preparing six-point calibration curves, spiking urine before and after extraction. To calculate recovery, calculated concentration must first be computed by interpolating areas obtained from the postextracted spiked samples into the pre-extracted spiked urine calibration curve. Then, the ratio analyte concentration/IS concentration was plotted against the calculated concentration explained above and a linear regression model was applied. The slope of the linear regression multiplied by 100 represents the analyte recovery.

Recovery (IS-normalized) in urine for IX, XN, and 8PN was 97.1% ± 0.03, 105.8% ± 0.05, and 105.4% ± 0.05, respectively (n = 10), and recovery of taxifolin (IS) was 100% ± 0.05. Recovery parameters of the new method developed in this work were greater compared to the other published methods, which reported low recoveries for IX, XN, and 8PN. Hanske et al.[5] obtained a recovery for XN in urine of 68%, and Wyns et al.[19] reported recovery values for XN, 8PN, and IX in urine of 74%, 78%, and 91%, respectively.

The matrix effect was evaluated with the method previously described by Matuszewski et al.[12] by spiking urine after SPE extraction and comparing the analyte area responses with neat standards in the mobile phase. Therefore, the closer the matrix effect factor is to 100%, the lower the matrix effect.

The optimized Oasis MCX extraction gave matrix effect factors for IX, XN, and 8PN of 110% ± 8.2, 82% ± 13, and 94% ± 15, respectively, leading to the elimination of urine interferences and avoiding signal suppression. Process efficiency, which depends on the matrix effect factors and recovery,[12] was high, with values of 93%, 112%, and 111% for IX, XN, and 8PN, respectively.

Selectivity. Blank urine samples from different volunteers (n = 6) were extracted as described in SPE Extraction Method, and the MRM chromatograms obtained were evaluated for the presence of interferences in the retention time of analytes and internal standard. Blank urine was then spiked with known low concentrations (5 $\mu g\ L^{-1}$) to ensure selectivity of the method at the lower limit of quantification.

Selectivity of the method was confirmed through the absence of endogenous peaks in MRM chromatograms at the same retention time as the analytes and internal standard in six human urine samples. The method was thus shown to be selective in the presence of analytes at low concentrations and

dx.doi.org/10.1021/ac4007733 | Anal. Chem. 2013, 85, 5547−5554

Table 4. Validation Results: Recovery, Matrix Effect, LOD, LOQ, Intraday and Interday Accuracy and Precision[a]

	recovery (%)	matrix effect (%)	LOD (μg L^{-1})	LOQ (μg L^{-1})	concn (μg L^{-1})	intraday 1		intraday 2		intraday 3		interday	
						accuracy (%)	precision (% CV)	accuracy (%)	precision (% CV)	accuracy (%)	precision (% CV)	accuracy (%)	precision (% CV)
isoxanthohumol	97.1 ± 0.03	110 ± 8.2	0.03	3	5	0	6.1	8.2	10.3	10.1	7.3	6.1	8.7
					15	2.2	2.8	0.7	6.9	9.0	6.4	3.1	7.0
					30	3.1	10.9	4.4	9.1	7.1	3.6	5.8	6.4
xanthohumol	105.8 ± 0.05	82 ± 13	0.07	5	5	14.4	6.4	0.7	12.8	13.0	7.5	8.9	10.5
					15	0.5	10.3	2.7	10.2	14.6	11.5	4.1	12.4
					30	10.3	13.9	10.1	10.7	12.7	13.7	6.3	12.6
8-prenylnaringenin	105.4 ± 0.04	94 ± 15	0.03	5	5	9.0	4.04	8.7	5.6	14.5	4.3	1.5	11.6
					15	7.5	8.8	6.9	7.7	10.1	9.3	5.4	6.6
					30	7.9	4.8	2.9	10.1	7.0	5.5	6.1	8.7

[a]LOD, limit of detection; LOQ, limit of quantification.

was able to discriminate between analytes and other components in urine.

Linearity, LOD, and LOQ. Calibration curves showed linear responses for all analytes within the range of 3 μg L^{-1} to 1000 μg L^{-1} and the corresponding regression correlation coefficients (r^2) were all >0.99. LOD for IX, XN, and 8PN were 0.03 μg L^{-1}, 0.07 μg L^{-1}, and 0.03 μg L^{-1}, respectively, in urine samples. LOQ were set for IX at 3 μg L^{-1} and 5 μg L^{-1} for 8PN and XN. The LOD and LOQ obtained in this work were lower than previously published methods. Wyns et al.[19] obtained an LOD of 0.2, 0.6, and 0.4 μg L^{-1} for IX, XN, and 8PN, respectively, in urine, using an HPLC−APCI-MS method. Avula et al.[20] reported a LOD of 130 μg L^{-1} in urine for XN, working with an HPLC−DAD. However, the method described in the present work provided a 10-fold lower LOD.

Stability. Postpreparative stability assay showed no significant reduction of analyte concentrations in the urine matrix after 24 h in the autosampler (Figure 2) at both low and high concentrations when using the optimized conditions obtained in this work suggesting that the analytes were stable under pH 7.0 and in the presence of ascorbic acid

Freeze and thaw stability assay show a signal decline of 18.4% for 8PN and 31.4% for XN after the third freeze and thaw cycle. However, no significant changes in concentration were observed for IX during the freeze and thaw cycles. Therefore, in prenylflavanoids analysis, samples cannot be refrozen for further analysis, especially when quantifying 8PN and XN.

Analysis of Beer Samples. Beer foam was removed by ultrasonication to degasificate the sample and filter. Then 20 μL of beer was injected into the LC−MS/MS system as described in LC−ESI-MS/MS equipment to evaluate the quantity of prenylflavanoids volunteers received when consuming a moderate dose of beer (330 mL).

The most abundant prenylflavanoid found in beer samples was IX, since XN isomerizes into IX during the brewing process.[21] The mean concentrations of IX and 8PN were found to be 460.7 ± 26 μg L^{-1} (n = 3) and 55.4 ± 0.01 μg L^{-1} (n = 3), respectively. XN was not detected in the beer samples provided to the volunteers. These results are in accordance with other papers.[22,23]

Analysis of Human Urine Samples. Urine samples from volunteers were analyzed using the method developed in this work to identify and quantify IX, XN, and 8PN after the consumption of a single moderate dose of beer (330 mL). Prenylflavanoids were identified by comparing their MRM transitions and the retention time of authentic standards (Figure 3). Analyte quantification was performed using six-point $1/x$ weighted calibration curves (IS-normalized) with standards between 0 and 40 μg L^{-1} and taxifolin as the internal standard.

IX concentration in human urine showed a significant increase (p < 0.001) 8 h after the beer consumption in human volunteers. 8PN was detected in the urine of all participants, even after the washout period, probably because 8PN is a microbial metabolite of IX. A delayed conversion of IX into 8PN has been shown, since IX has to reach the distal colon, possibly after absorption and enterohepatic recirculation, which can take up to 48 h.[4] Schaefer et al.[24] demonstrated a slow 8PN urinary excretion (up to several days) after beer consumption. Thus, a 4-day washout period may not be enough to eliminate urinary traces of 8PN. However, in all cases the level of 8PN in urine was between the LOD and LLOQ. XN was not detected in the urine of any participant. So the only compound in the

dx.doi.org/10.1021/ac4007733 | Anal. Chem. 2013, 85, 5547−5554

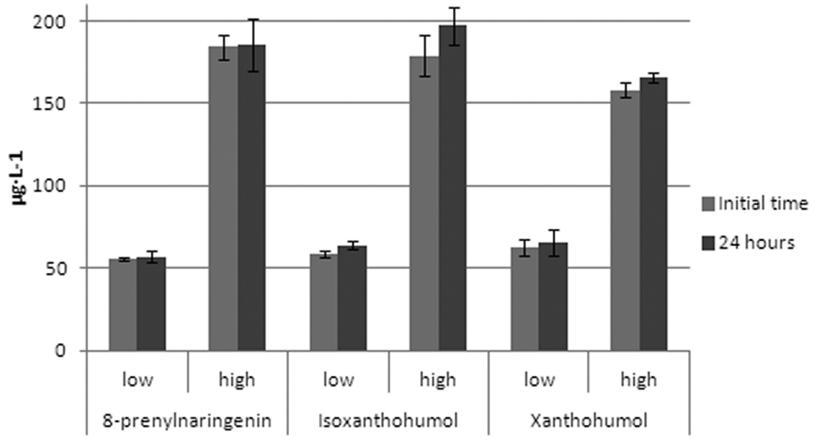


Figure 2. 8PN, IX and XN postpreparative stability. Mean concentrations (μg L^{-1}) of 8-prenylnaringenin, isoxanthohumol and xanthohumol recovered at initial time (t = 0) and at 24 h. Two standard concentrations (50 and 150 μg L^{-1}) were prepared in urine and injected at the initial time and after 24 h to evaluate the stability of the analytes under neutral conditions (pH 7.0) and in the presence of ascorbic acid as an antioxidant.

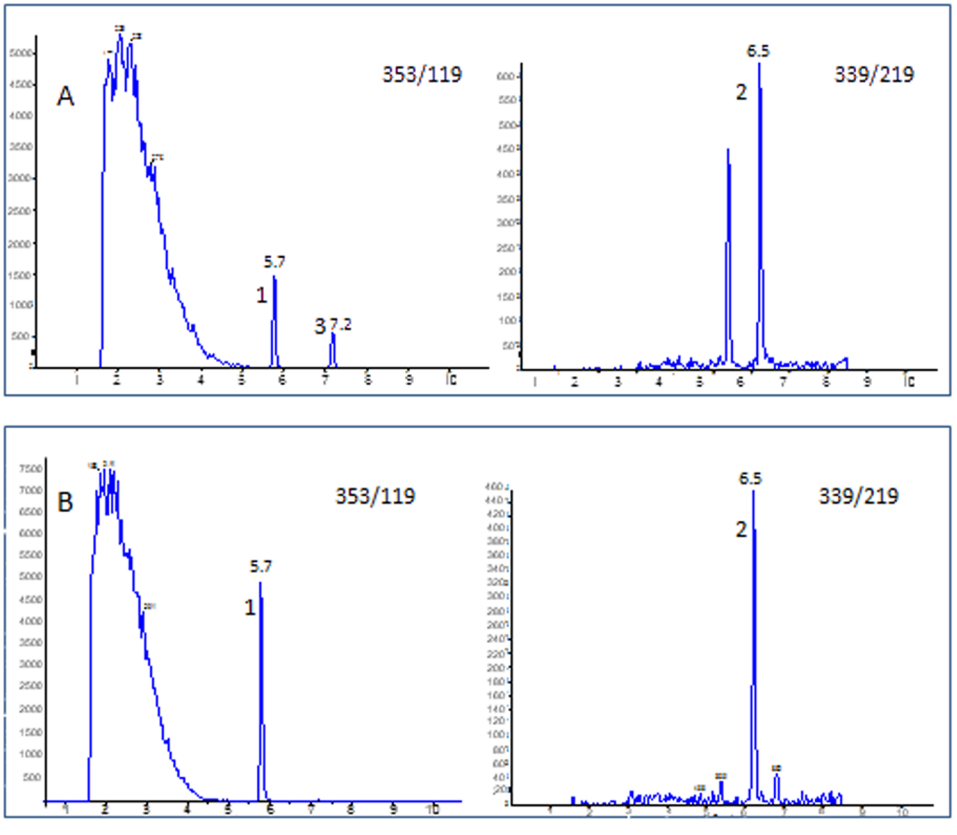


Figure 3. Multiple reaction monitoring (MRM) chromatograms: (A) spiked urine with standards (1 μg L^{-1}); (B) male volunteer urine 8 h after the consumption of 330 mL of beer; (1) isoxanthohumol, (2) 8-prenylnaringenin, (3) xanthohumol.

beer that allowed discrimination between beer and nonbeer consumers was IX. The mean urinary excretion of IX after a moderate dose of beer was 2.48 μg IX g creatinine^{-1} (95% CI, 1.90−3.06 μg IX g creatinine^{-1}).

CONCLUSIONS

In this work, a new method to analyze IX, XN, and 8PN by LC−ESI-MS/MS was developed and fully validated. Several methods to analyze prenylflavanoids in different biological matrices have been published,[4,19,20,25−28] but only a few are validated. Most of these methods use reverse phase SPE (C_{18}) for extraction.[25−27] To our knowledge, this is the first time that a cation-exchange sorbent has been assessed to improve analyte recovery and reduce the matrix effect caused by urine components. In this work, it was demonstrated that using a mixed-mode cation-exchange sorbent such as Oasis MCX reduces signal suppression of the urine matrix due to its ability to remove interferences from urine more thoroughly without compromising analyte recovery. Consequently, the LOD and LOQ obtained were lower than those of previously published methods.

Furthermore, after evaluating the pH effect on reproducibility, accuracy, precision, and stability in the analysis of IX, XN, and 8N, it was concluded that a reconstitution medium and

dx.doi.org/10.1021/ac4007733 | Anal. Chem. 2013, 85, 5547−5554

mobile phase at pH 7.0 is required for the analysis of prenylflavanoids to minimize analyte degradation. In contrast, all methods published until now have used acidic mobile phases and reconstitution media after SPE.[4,19,20,25−28] The addition of an antioxidant such as ascorbic acid to the reconstitution media helped to maintain analytes stable for at least 24 h.

The described SPE-LC−ESI-MS/MS method allowed IX, XN, and 8PN to be determined in human urine after beer consumption. The significant improvement in specificity, sensitivity, and recovery achieved will enable the method to be applied in studies with a large number of samples, such as clinical and epidemiological trials.

Finally, the clinical study performed in this work demonstrated that IX is the only prenylflavanoid that can discriminate between no-beer and beer consumption after the intake of a moderate dose of beer (330 mL).

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. All authors contributed equally.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This study was supported by The European Foundation for Alcohol Research (ERAB) EA 11 17. We express our gratitude for financial support from CICYT(AGL2010-22319-C03), the Spanish Ministry of Science and Innovation (MICINN), and CIBEROBN of the Instituto de Salud Carlos III. Paola Quifer-Rada and Gemma Chiva-Blanch would like to thank the Manuel de Oya fellowship from Centro de Información Cerveza Salud y Nutrición. Miriam Martínez-Huélamo thanks the predoctoral program of MICINN.

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4.2.2. Publication 3. Is enzymatic hydrolysis a reliable analytical strategy to quantify glucuronidated and sulfated polyphenols metabolites in human fluids?

Paola Quifer-Rada, Miriam Martínez-Huélamo, Rosa M. Lamuela-Raventos. *Food and Function*, submitted

Summary:

Polyphenol consumption has been associated with a reduced risk of several chronic diseases. Phenolic compounds are present in human fluids (plasma and urine) mainly as glucuronidated and sulfated metabolites. However, quantification of conjugated polyphenol metabolites in biological samples is hampered by the unavailability of standards. Thus, up to now, the most commonly applied analytical method is enzymatic hydrolysis, which breaks the *O*-glucuronide or *O*-sulfate bond and quantifies the polyphenol precursor. Enzymatic hydrolysis procedures vary in enzyme concentration, pH and temperature; however, there is a lack of knowledge about the stability of polyphenols in their free form during the process. The aim of this work was to analyze the stability of 12 representative non-conjugated polyphenols (including seven phenolic acids, two flavonoids and three prenylflavanoids) during enzymatic hydrolysis in order to assess the suitability of this analytical procedure, using three different concentrations of β-glucuronidase/sulfatase from *Helix pomatia.*

Synthetic urine was spiked with a mix of polyphenol standards. In order to assess the recovery of polyphenols in their free form, a general hydrolysis protocol was performed using three different concentrations of enzymes. Additionally, two control samples were prepared using the same spiked synthetic urine: one control underwent the same procedure but without an added enzyme to evaluate if the temperature (37 ºC) and pH (5.0) conditions used for the hydrolysis affected the recoveries, while the second control was prepared without following the hydrolysis procedure. After hydrolysis of the samples, an SPE step was applied to extract the analytes and samples were analyzed by LC-ESI-MS/MS. Analyte recovery during enzymatic hydrolysis was calculated to measure the effectiveness of the procedure

The results suggested that the temperature (37 ºC) and pH (5.0) regularly used in enzymatic hydrolysis only affected prenylflavanoid recovery. In contrast, enzyme addition resulted in a significantly lower recovery of almost all the compounds ($p<0.05$), with 3,3-hydroxyphenylpropionic acid, 3-hydroxyphenylacetic acid, caffeic acid, and isoferulic acid suffering a notable reduction of approximately 50%. Quercetin and naringenin recovery decreased by almost 60%. Furthermore, the decrease in analyte recovery was significantly

correlated with an increase in enzymatic concentration. Thus, enzymatic hydrolysis might underestimate levels of polyphenol metabolites since it can affect the recovery of polyphenols in their free form generated in the sample during the hydrolysis procedure as well as of the original polyphenols.

Is enzymatic hydrolysis a reliable analytical strategy to quantify glucuronidated and sulfated polyphenol metabolites in human fluids?

Journal:	*Food & Function*
Manuscript ID	FO-COM-04-2017-000558
Article Type:	Communication
Date Submitted by the Author:	11-Apr-2017
Complete List of Authors:	Quifer-Rada, Paola; University of Barcelona, Martínez-Huélamo, Miriam; University of Barcelona, Nutrition, Food Science and Gastronomy Lamuela-Raventos, Rosa M.; University of Barcelona, Nutrition, Food Science and Gastronomy

Food & Function

2015 Impact Factor: 2.676
www.rsc.org/foodfunction

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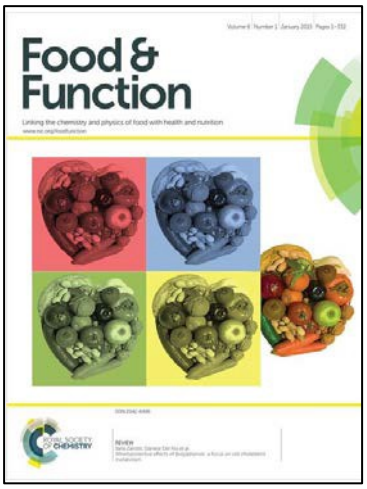

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Best wishes,

Philippa Hughes
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Professor Kevin Croft
Editor-in-Chief

Is enzymatic hydrolysis a reliable analytical strategy to quantify glucuronidated and sulfated polyphenol metabolites in human fluids?

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ABSTRACT

Polyphenol consumption has been associated with a reduced risk of several chronic diseases. Phenolic compounds are present in human fluids (plasma and urine) mainly as glucuronidated and sulfated metabolites. Up to now, due to the unavailability of standards, enzymatic hydrolysis has been the method of choice in analytical chemistry to quantify these phase II phenolic metabolites. Enzymatic hydrolysis procedures vary in enzyme concentration, pH and temperature; however, there is a lack of knowledge about the stability of polyphenols in their free form during the process. In this study, we evaluated the stability of 7 phenolic acids, 2 flavonoids and 3 prenylflavanoids in urine during enzymatic hydrolysis to assess the suitability of this analytical procedure, using three different concentrations of β-glucuronidase/sulfatase enzymes from *Helix pomatia*. The results indicate that enzymatic hydrolysis negatively affected the recovery of the precursor and free-form polyphenols present in the sample. Thus, enzymatic hydrolysis does not seem an ideal analytical strategy to quantify glucuronidated and sulfated polyphenol metabolites.

KEYWORDS: enzymatic hydrolysis, polyphenol metabolites, recovery, quantification, urine

1. Introduction

Polyphenol consumption has been associated with a reduced risk of cardiovascular disease and cancer in epidemiological[1–4] and clinical intervention studies.[5–8] During intestinal absorption and passage to the liver, polyphenols undergo phase II metabolism to glucuronidated and/or sulfated forms, whereas phase I metabolism (oxidation and reduction reactions) appears to be minor. These phase II metabolites are excreted back to the intestine through enterohepatic circulation, where they can be re-absorbed or metabolized by gut microbiota in the colon or excreted into the urine[9].

Most flavonoids are not very well absorbed in the intestinal tract and are also metabolized by gut microbiota into phenolic acids such as 4-hydroxybenzoic, protocatechuic and vanillic acids, which can be converted to hippuric acid derivatives upon absorption[10-14] or conjugated to their glucuronide and sulfate forms.[15-19]

Identification and quantification of conjugated polyphenol metabolites in biological samples is hampered by the unavailability of standards. The most commonly applied analytical method is enzymatic hydrolysis, which breaks the *O*-glucuronide or *O*-sulfate bond and quantifies the polyphenol precursor.[20-34] Table 1 shows different enzymatic treatments applied to quantify phenolic compounds in urine samples. There are diverse enzymes for conducting hydrolysis but the most generally used enzyme in polyphenols hydrolysis is *Helix pomatia*. However, to date, studies using this approach have not evaluated the possible degradation of the polyphenol precursor during this step. The aim of this work was to analyze the stability of 12 representative non-conjugated polyphenols during enzymatic hydrolysis in order to assess the suitability of this analytical procedure, using three different concentrations of β-glucuronidase/sulfatase from *Helix pomatia.*

2. Experimental

2.1 Reagents and materials

Caffeic, ferulic, isoferulic and hippuric acids, naringenin and quercetin (97-99% purity) and type H-2 β-glucuronidase from *Helix pomatia* were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Isoxanthohumol, 8-prenylnaringenin and xanthohumol (97-99% purity) were purchased from Enzo Life Science (Lausen, Switzerland). 3-(3-hydroxyphenyl)propionic and 3,4-dihydroxyphenylacetic acids were purchased from Extrasynthese (Genay, France).

Methanol (MeOH) and acetonitrile (MeCN) of HPLC grade were obtained from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade acetic acid, formic acid and ammonium acetate were purchased from Panreac Quimica S.A (Barcelona, Spain). Ultrapure water (MilliQ) was generated by the Millipore System (Bedford, USA). Solid phase extraction (SPE) cartridges Oasis HLB and MCX 96-well plate 30 µm (30 mg) were obtained from Waters (Milford, MA, USA).

Synthetic human urine was prepared by dissolving 0.65 g L^{-1} of $CaCl_2 \cdot 2H_2O$, 0.65 g L^{-1} $MgCl_2 \cdot H_2O$, 4.6 g L^{-1} NaCl, 2.3 g L^{-1} Na_2SO_4, 0.65 g L^{-1} $Na_3C_6H_5O_7 \cdot 2H_2O$, 2.8 g L^{-1} KH_2PO_4, 1.6 g L^{-1} KCl, 1.0 g L^{-1} NH_4Cl, 25 g L^{-1} urea, and 1.1 g L^{-1} creatinine in Milli-Q water.

2.2 Enzymatic hydrolysis

The enzyme most commonly used for polyphenolic hydrolysis, β-glucuronidase/sulfatase from *Helix pomatia*, was used to evaluate the suitability of the enzymatic hydrolysis strategy for quantifying glucuronidated/sulfated phenolic metabolites. Enzymatic hydrolysis was assessed with 7 phenolic acids, 2 flavonoids and 3 prenylflavanoids in urine. Figure 1 shows the chemical structures of the 12 phenolic compounds studied.

Synthetic urine was spiked with a mix of polyphenol standards at 500 µg L^{-1} (phenolic acids and flavonoids) and 100 µg L^{-1} (prenylflavanoids). In order to assess the recovery of polyphenols in their free form, a general hydrolysis protocol was performed using three different concentrations of enzymes used in previously published studies.[26-28] Briefly, 1 mL of the spiked synthetic urine was adjusted to pH 5.0 by adding 400 µL of 2 M sodium acetate buffer. Then, 60 µL (3493 units of β-glucuronidase, 129 units of sulfatase), 80 µL (4595 units of β-glucuronidase, 131 units of sulfatase) or 130 µL (7222 units of β-glucuronidase, 135 units of sulfatase) were added to the urine and incubated for 1 hour at 37 ºC in a heating bath. Additionally, two control samples were prepared using the same spiked synthetic urine: one control underwent the same procedure but without an added enzyme to evaluate if the temperature (37 ºC)

and pH (5.0) conditions used for the hydrolysis affected the recoveries, while the second control was prepared without following the hydrolysis procedure. All samples were prepared in triplicate and were quantified using a 6-point calibration curve prepared in synthetic urine.

2.3 Solid phase extraction

Oasis HLB 96-well plates were used to extract phenolic acids and flavonoids following the previously described method.[35] 1 mL of MeOH and 1 mL of 1.5 M formic acid were added to activate the cartridges. 1 mL of the hydrolysed urine was loaded, and sample cleanup was performed with 1 mL of 1.5 M formic acid followed by 1 mL of 5% MeOH solution. Phenolic compounds were then eluted with 1 mL of MeOH acidified with 0.1% formic acid (v/v). The elution fraction obtained was evaporated to dryness at room temperature under a stream of nitrogen. 100 µL of water acidified with 0.1% formic acid (v/v) was added to dissolve the residue to be injected into the LC system.

Prenylflavanoids were extracted following the method previously described based on Oasis MCX 96-well plates.[36] Briefly, cartridges were equilibrated with 1 mL of MeOH and 1 mL of 5 mM ammonium acetate buffer adjusted to pH 5.0. Then 1 mL of the hydrolysed urine was loaded into the cartridges. Samples were washed with 1 mL of MeOH:5 mM of aqueous ammonium acetate buffer at pH 5 (1:1, v/v) solution and eluted with 1 mL of MeOH. The eluate was evaporated to dryness under a gentle stream of N_2 and the residue was reconstituted with 100 µL of 5 mM ammonium bicarbonate buffer at pH 7 containing 20 mg L^{-1} of ascorbic acid.

2.4 LC-ESI-MS/MS methodology

LC analyses were performed using an HP Agilent 1100 system equipped with a quaternary pump and a refrigerated autosampler (Waldbronn, Germany) coupled to an API 3000 triple-quadrupole mass spectrometer (Sciex, Framinghan, MA, USA) with a turbo ion spray source working in negative mode. Chromatographic separation was performed with a Phenomenex Luna C_{18} column, 50 mm x 2.0 mm i.d, 5 µm (Torrance, CA, USA) and coupled to a pre-column Phenomenex security guard C18 (4 x 2 mm i.d.). The system and acquisition data were controlled by Analyst v 1.4.2 software supplied by Sciex (Framinghan, MA, USA).

Prenylflavanoids were eluted with a nonlinear gradient using 5 mM of ammonium bicarbonate buffer adjusted to pH 7.0 as the aqueous mobile phase (A) and acetonitrile and methanol as organic phases in a proportion of 1:1. The gradient applied was: 0 min, 95% A; 0-1 min, 87% A; 1-2.5 min, 60% A; 2.5-5 min, 30% A; 5-7 min, 0% A; 7-8 min, 0% A; 8-8,5 min, 95% A; 8,5-11 min, 95% A. The injection volume was 20 μL and the flow rate was 600 μL min^{-1}.

Phenolic acids and flavonoids were analysed using water as mobile phase A and MeCN as mobile phase B, with 0.1% formic acid in both solvents. The flow rate was 600 μL min^{-1}, and the gradient used was: 0 min, 95% A; 2 min, 75% A; 10 min, 10% A; 11 min, 0% A; and 12 min, 0% A, followed by a 5-min re-equilibration step. 20 μL aliquots of the extracts were injected in the LC-MS/MS system.

Multiple Reaction Monitoring (MRM) mode was used to quantify analytes. The turbo ion spray source was used in negative mode with the following settings: capillary voltage -4000 V, nebulizer gas (N_2) 10 (arbitrary units), curtain gas (N_2) 12 (arbitrary units), and drying gas (N_2) heated to 400 ºC and introduced at a flow-rate of 6000 mL min^{-1}. Previously described MRM parameters were used.[35,36]

Analyte recovery was measured to evaluate the effectiveness of enzymatic hydrolysis. Statistical differences between enzymatic and non-enzymatic treated samples were determined with a Mann-Whitney test.

3. Results and discussion

Analyte recovery during enzymatic hydrolysis was calculated to measure the effectiveness of the procedure. Table 2 summarises the recoveries obtained with the three different concentrations of the β-glucuronidase/sulfatase enzymes studied.

The results suggested that the temperature (37 ºC) and pH (5.0) regularly used in enzymatic hydrolysis only affected prenylflavanoid recovery (see Table 2). It has been reported elsewhere that prenylflavonoids are extremely unstable and need specific analytical conditions to avoid degradation,[36] so they may be altered during enzymatic hydrolysis. In contrast, enzyme addition resulted in a significantly lower recovery of almost all the compounds (p<0.05), with 3,3-hydroxyphenylpropionic acid, 3-

hydroxyphenylacetic acid, caffeic acid, and isoferulic acid suffering a notable reduction of approximately 50%. A similar affect was observed with the two flavonoids studied, quercetin and naringenin, whose recovery decreased by almost 60%. Furthermore, the decrease in analyte recovery was significantly correlated with an increase in enzymatic concentration, suggesting that the higher the concentration, the greater the degradation of polyphenols in their free form, with the exception of isoxanthohumol, which was considerably affected by the hydrolysis conditions (temperature and pH) but not by the enzymatic activity. Ferulic acid was the only polyphenol unaffected by the presence of the enzyme or the hydrolysis conditions, as shown in Table 1.

This study provides evidence that enzymatic hydrolysis might underestimate levels of polyphenol metabolites since it can affect the recovery of polyphenols in their free form generated in the sample during the hydrolysis procedure as well as of the original polyphenols.

4. Conclusions

In conclusion, as an analytical procedure that breaks down sulfate and glucuronide bonds, enzymatic hydrolysis might be overly aggressive, thereby compromising recovery of the hydrolyzed polyphenols and their free forms found in the sample. The results of our study question the suitability of enzymatic hydrolysis as an analytical strategy for evaluating glucuronidated and sulfated polyphenol metabolites.

ACKNOWLEDGMENT

This work was supported in part by CICYT (AGL2013-49083-C3-1-R and AGL2016-79113-R), the Instituto de Salud Carlos III, ISCIII (CIBEROBN) from the Ministerio de Economía y Competitividad (MEC) (AEI/FEDER, UE), the Generalitat de Catalunya (GC) 2014 SGR 773 and The European Foundation for Alcohol Research (ERAB) EA 1324, EA 1514, EA 1515 and EA1517.

Paola Quifer-Rada is grateful for the predoctoral fellowships awarded by the Generalitat de Catalunya (FI-DRG).

Miriam Martínez-Huélamo and Paola Quifer-Rada declare equal contribution to the work. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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285 **TABLES**

286 **Table 1. Examples of enzymatic treatments in phenolic compounds in urine samples**

Analytes	Buffer	pH	Enzyme	Incubation time	Temperature (ºC)	Ref.
Catechin, daidzein, dihydrodaidzein, dihydrogenistein, epicatechin, glycitein, hesperetin, 8-hydroxydaidzein, hydroxybiochanin A, myricitin, quercetin, apigenin, biochanin A	Sodium Acetate	4.66	β-glucuronidase/sulfatase from *Helix pomatia*	18 h	37 °C	20
Quercetin, fisetin	Sodium Acetate/Ascorbic acid	5	β-glucuronidase from bovine liver/sulfatase from *Aerobacter aerogenes*	1 h	37 °C	21
Hesperetin	Sodium Acetate	5.5	β-glucuronidase/sulfatase from *Helix pomatia*	overnight	37 °C	22
Genistein, daidzein, glycitin, puerarin, biochanin A, catechin, epicatechin, rutin, hesperidin, neohesperidin, quercitrin, hesperetin	Sodium Acetate	4.66	β-glucuronidase/sulfatase from *Helix pomatia*	18 h	37 °C	23
Pinobanksin 5-methyl ether, pinobanksin, kaempferol, chrysin, pinocembrin, galangin	Acetate	4.5	β-glucuronidase/sulfatase	20 min	37 °C	24
Epicatechin-*O*-glucuronide, *O*-methyl(epi)catechin-*O*-glucuronide, epicatechin sulfate, *O*-methyl(epi) catechin sulfate, 5-(dihydroxyphenyl)-c-valerolactone glucuronide, 5-(dihydroxyphenyl)-c-valerolactone sulfate, 5-(hydroxy-methoxy-phenyl)-c-valerolactone glucuronide, 5-(hydroxy-methoxy-phenyl)-c-valerolactone sulfate, naringenin-*O*-glucuronide, isorhamnetin-*O*-glucuronide, isorhamnetin sulfate, 5-(dihydroxyphenyl)-c-valerolactone, 5-(hydroxyphenyl) -c-valerolactone, 3,4-dihydroxyphenylpropionic acid, 3-hydroxyphenylpropionic acid, 3,4- dihydroxy phenylacetic acid, 3-hydroxyphenylacetic acid, 4-hydroxy-3-methoxy-phenylacetic acid, phenylacetic acid, m-coumaric acid, p-coumaric acid, caffeic acid, ferulic acid, 3-hydroxy-4-methoxy-cinnamic acid, 3-	Sodium Acetate	4.9	β-glucuronidase/sulfatase from *Helix pomatia*	45 min	37 °C	25

hydroxybenzoic acid, 4-hydroxybenzoic acid, protocatechuic acid, vanillic acid, 4-hydroxyhippuric acid						
3,4-Dihydroxyphenylpropionic acid, 4-hydroxyphenylpropionic acid, m-coumaric acid, p-coumaric acid, caffeic acid, ferulic acid, 3,4-dihydroxyphenylacetic acid, 3-methoxy-4-hydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, phenylacetic acid, protocatechuic, acid, vanillic acid, 4-hydroxybenzoic acid, 3-hydroxybenzoic acid, 4-hydroxyhippuric acid, hippuric acid, 4-*O*-methylgallic acid, enterodiol, enterolactone, epicatehin, procyanidin B2	Acetic Acid	4.9	β-glucuronidase/sulfatase from *Helix pomatia*	45 min	37 °C	26
Hydroxytyrosol, tyrosol, pinoresinol, luteolin,acetoxypinoresinol, apigenin, oleuropein aglycone, ligstroside aglycone	Sodium Acetate	5	β-glucuronidase from *Helix pomatia*	4 h	37 °C	27
Oleuropein, hydroxytyrosol, 3-(4-hydroxyphenyl)-propanol, 3-*O*-methyl-hydroxytyrosol, 4-methylcatechol, 3-(4-hydroxyphenyl)-propanol	Sodium Acetate	5.2	β-glucuronidase from *Helix pomatia*	17 h	37 °C	28
Epicatechin-*O*-glucuronide, *O*-methyl(epi)catechin-*O*-glucuronide, epicatechin sulfate, *O*-methyl(epi) catechin sulfate, naringenin-*O*-glucuronide, isorhamnetin-O-glucuronide, isorhamnetin sulfate, 5-(dihydroxyphenyl)-c-valerolactone glucuronide, 5-(dihydroxyphenyl)-c-valerolactone sulfate, 5-(hydroxy-methoxy-phenyl)-c-valerolactone glucuronide, 5-(hydroxy-methoxy-phenyl)-c-valerolactone sulfate, 3,4-dihydroxyphenylpropionic acid, 3-hydroxyphenylpropionic acid, 3,4- dihydroxy phenylacetic acid, 3-hydroxyphenylacetic acid, 3,4-dihydroxy phenylacetic acid, 3-hydroxyphenylacetic acid, 4-hydroxy-3-methoxy-phenylacetic acid, phenylacetic acid, m-coumaric acid, p-coumaric acid, caffeic acid, ferulic acid, 3-hydroxy-4-methoxy-cinnamic acid, 3- hydroxybenzoic acid, 4-	Sodium Acetate	4.9	β-glucuronidase from *Helix pomatia*	45 min	37 °C	29

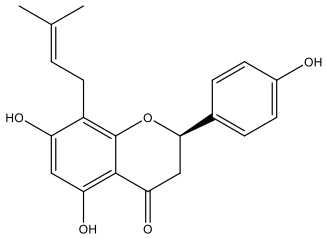
hydroxybenzoic acid, protocatechuic acid, vanillic acid, 4-hydroxyhippuric acid						
Genistein, diadzein, glycetein, dihydrodaidzein, *O*-desmethylangolensin, dihydrogenistein	Ammonium Acetate	5	β-glucuronidase from bovine liver/sulfatase from *Helix pomatia*/sulfatase from *abalone entrails*	3 h	37 °C	30
Genistein	Ammonium Acetate	4.6	β-glucuronidase	24 h	37 °C	31
Genistein, diadzein, biochanin A, glycetein, equol, dihydrodaidzein, *O*-desmethylangolensin dihydrogenistein	Ammonium Acetate	5	β-glucuronidase from bovine liver/sulfatase from *abalone entrail*/sulfatase/β-glucuronidase from *Helix pomatia*	3 h	37 °C	32
Luteolin, apigenin	Sodium Acetate	5	β-glucuronidase from *E.coli*/sulfatase from *Helix pomatia*	2 h	37 °C	33
3-hydroxybenzoic acid, 4-hydroxybenzoic acid, hippuric acid, vanillic acid, phenylacetic acid, 3-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid, 3-hydroxyphenylpropionic acid, 3,4-dihydroxyphenylpropionic acid, caffeic acid, ferulic acid, 3-hydroxyphenylvaleric acid, 3-methoxy,4-hydroxyphenylvalerolactone, 3,4-dihydroxyphenylvalerolactone, catechin, 3'-*O*-methylcatechin	Sodium Acetate	5	β-glucuronidase from *Helix pomatia*/sulfatase from *Patella vulgata*	3 h	37 °C	34

287 **Table 2.** Recoveries of the polyphenol precursors after enzymatic hydrolysis with three different
288 concentrations of β-glucuronidase/sulfatase.

	Recovery (%) ± SD				
	*0 MU β-glucuronidase**	*3.5 MU β-glucuronidase*	*4.6 MU β-glucuronidase*	*7.2 MU β-glucuronidase*	*P*
8-prenylnaringenin	77.4±4.9	60.4±5.7	67±1.4	44.5±1.3	0.01
Isoxanthohumol	50.2±2.1	44±7.4	50.7±2.8	37.9±2.1	0.30
Xanthohumol	62.9±13.3	23.8±4.3	25.4±12.6	44.1±3.6	0.01
3,3-hydroxyphenylpropionic acid	120.5±1.2	78.6±2.2	63.1±1.7	51.8±5	0.01
3,4-dihydroxyphenylacetic acid	64.6±2.8	64.5±8.4	55.7±6.4	45.1±3.1	0.04
3-hydroxyphenylacetic acid	108.5±9.2	57.1±5	45.1±2.7	46.6±2.8	0.01
Caffeic acid	111±3.8	62.1±0.6	50±2.2	46.4±3.2	0.01
Ferulic acid	110±1.9	106.1±5.3	109.1±0.4	116.7±3.3	0.90
Hippuric acid	98.4±2.7	77±0.9	80.6±3.9	80.4±5.8	0.04
Isoferulic acid	106.4±3.7	73.8±4.8	59.8±4.9	46±5.9	0.01
Naringenin	106.7±15.4	84.4±4.4	84.1±8.3	41.6±5.8	0.02
Quercetin	108±4.8	49.1±4.4	50.9±6.5	35.8±1.4	0.03

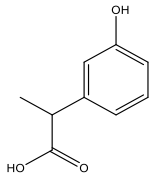
289 *Hydrolysis conditions such as pH and temperature are evaluated.

Prenylflavanoids

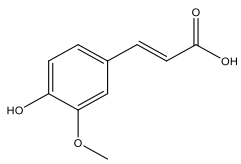


8-Prenylnaringenin

Phenolic acids

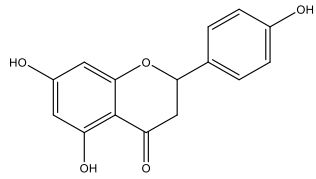


3,3-Hydroxyphenyl-propionic acid

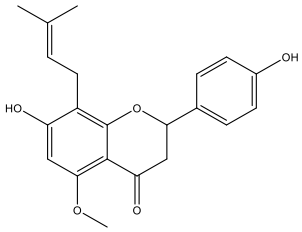


Ferulic acid

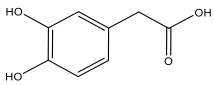
Flavonoids



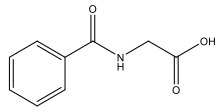
Naringenin



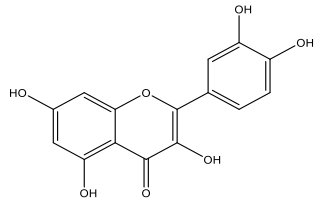
Xanthohumol



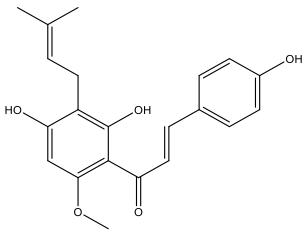
3,4-Dihydroxyphenyl-acetic acid



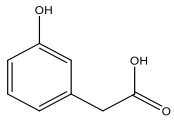
Hippuric acid



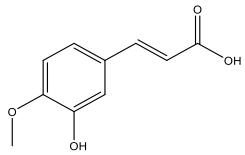
Quercetin



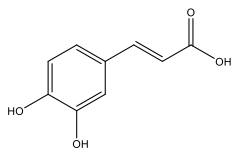
Isoxanthohumol



3-Hydroxyphenyl-acetic acid



Isoferulic acid



Caffeic acid

4.2.3. Publication 4. Urinary isoxanthohumol is a specific and accurate biomarker of beer consumption.

Paola Quifer-Rada, Miriam Martínez-Huélamo, Gemma Chiva-Blanch, Olga Jáuregui, Ramon Estruch, and Rosa M. Lamuela-Raventós. *Journal of Nutrition*, 2014 ;144(4):484-8.

Summary:

In nutritional epidemiology, an accurate assessment of dietary exposure is crucial for studying the effect of diet on health. However, FFQ are liable to systematic bias, whereas biomarkers of food consumption are a powerful tool to obtain more objective measurements of dietary exposure and to monitor compliance in clinical trials. However, there are currently no available biomarkers for beer intake. In this work, we evaluated the effectiveness of urinary isoxanthohumol excretion as an accurate biomarker of beer consumption.

The suitability of beer prenylflavanoids to use as a specific and selective biomarker of beer consumption was evaluated in two different intervention clinical trials (studies 1 and 2) and in a cohort study (study 3). The study 1 was a the dose-response trial with 41 young volunteers (male and female, age 28±3 years) who consumed different doses of beer at night and a spot urine sample was collected the following morning. The doses of beer administered to male volunteers were: 1 beer (330 mL), 2 beers (660 mL) and 3 beers (990 mL), whereas women volunteers drank 1 beer (330 mL), 1.5 beers (495 mL) and 2 beers (660 mL). Study 2 was a clinical trial including 33 high cardiovascular risk men (age 61±7 years) who randomly received 30 g of ethanol/day as gin or beer, or an equivalent amount of polyphenols as non-alcoholic beer during 4 weeks. Study 3 was a free-living population from the PREDIMED cohort. A subsample of 46 volunteers of the PREDIMED (men and women, age 63±5 years) cohort was analyzed. In the three studies, prenylflavanoids were quantified in urine by SPE and LC-ESI-MS/MS following the method previously validated. Pearson linear correlation was used to evaluate the relationship between beer consumption and urinary excretion of prenylflavanoids. A ROC curve was assessed to calculate the sensitivity, specificity, accuracy and the ability to discriminate between positive and negative results of the proposed new biomarker.

Isoxanthohumol urinary excretion increased linearly with the beer dose size in male volunteers. A significant increase in isoxanthohumol excretion was found after consumption of beer and non-alcoholic beer for 4 weeks compared to the washout period ($P<0.001$), whereas no changes were observed after the gin period. ROC curves showed that isoxanthohumol is able to

discriminate between beer consumers and abstainers with a sensitivity of 67% and specificity of 100%; PPV= 70%, NPV = 100% in real-life conditions.

Nevertheless, 8-prenylnaringenin was detected in urine samples but under the limit of quantification and xanthohumol was not detected in any urine samples from volunteers.

In conclusion, isoxanthohumol in urine was found to be a specific and accurate biomarker of beer consumption and may be a powerful tool in epidemiological studies.

The Journal of Nutrition. First published ahead of print January 29, 2014 as doi: 10.3945/jn.113.185199.

The Journal of Nutrition
Nutritional Epidemiology

Urinary Isoxanthohumol Is a Specific and Accurate Biomarker of Beer Consumption[1–3]

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Abstract

Biomarkers of food consumption are a powerful tool to obtain more objective measurements of dietary exposure and to monitor compliance in clinical trials. In this study, we evaluated the effectiveness of urinary isoxanthohumol (IX) excretion as an accurate biomarker of beer consumption. A dose-response clinical trial, a randomized, crossover clinical trial, and a cohort study were performed. In the dose-response trial, 41 young volunteers (males and females, aged 28 ± 3 y) consumed different doses of beer at night and a spot urine sample was collected the following morning. In the clinical trial, 33 males with high cardiovascular risk (aged 61 ± 7 y) randomly were administered 30 g of ethanol/d as gin or beer, or an equivalent amount of polyphenols as nonalcoholic beer for 4 wk. Additionally, a subsample of 46 volunteers from the PREDIMED study (Prevención con Dieta Mediterránea) (males and females, aged 63 ± 5 y) was also evaluated. Prenylflavonoids were quantified in urine samples by liquid chromatography coupled to mass spectrometry. IX urinary recovery increased linearly with the size of the beer dose in male volunteers. A significant increase in IX excretion (4.0 ± 1.6 μg/g creatinine) was found after consumption of beer and nonalcoholic beer for 4 wk ($P < 0.001$). Receiver operating characteristic curves showed that IX is able to discriminate between beer consumers and abstainers with a sensitivity of 67% and specificity of 100% (positive predictive value = 70%, negative predictive value = 100% in real-life conditions). IX in urine samples was found to be a specific and accurate biomarker of beer consumption and may be a powerful tool in epidemiologic studies. This trial was registered at the International Standard Randomized Controlled Trial registry as ISRCTN72996101 (study 1), ISRCTN95345245 (study 2), and ISRCTN35739639 (study 3). J. Nutr. doi: 10.3945/jn.113.185199.

Introduction

In nutritional epidemiology, accurate assessment of dietary exposure is crucial for studying the effect of diet on health. Until now, the most common way of assessing the diet followed by participants in such studies has been by analyzing the results of self-reporting FFQs, but this method is liable to systematic bias because of factors such as age, gender, and social desirability and approval (1,2). In contrast, nutritional biomarkers have several advantages over self-reported data as indicators of dietary exposure, because they are more precise and provide more objective measurements (3). In this context, some of the previously used biomarkers of food intake have been based on the measurement of urinary concentration of polyphenols and related metabolites (4–6). Thus, urinary resveratrol metabolite measurements have been employed as biomarkers of wine consumption (4), and urinary 4-O-methylgallic acid and isoferulic acid have been proposed as potent biomarkers of tea and coffee intake (5). Moreover, data on nutrient intake based on biomarkers (i.e., polyphenol intake) correlates better with clinical measures (i.e., blood pressure) than data obtained by FFQ (7). However, there are currently no biomarkers available for beer intake.

The almost exclusive dietary sources of prenylflavonoids are hops and beer because female inflorescences of hops used in

[1] Supported by the European Foundation for Alcohol Research (ERAB) grants EA 1117 and EA 1324.

[2] Author disclosures: R. Estruch serves on the board of and receives lecture fees from the Research Foundation on Wine and Nutrition (FIVIN), serves on the boards of the Beer and Health Foundation and the European Foundation for Alcohol Research (ERAB), receives lecture fees from Cerveceros de España and Sanofi-Aventis, and received grant support through his institution from Novartis. R. M. Lamuela-Raventós serves on the board of and receives lecture fees from FIVIN, receives lecture fees from Cerveceros de España, and receives lecture fees and travel support from PepsiCo. These foundations had no involvement in the study design; the collection, analysis, and interpretation of data; the writing of the manuscript; or the decision to submit the manuscript for publication. P. Quifer-Rada, M. Martínez-Huélamo, G. Chiva-Blanch, and Olga Jáuregui, no conflicts of interest.

[3] Supplemental Figure 1 is available from the ''Online Supporting Material'' link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.

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Manuscript received September 13, 2013. Initial review completed October 13, 2013. Revision accepted January 8, 2014.
doi: 10.3945/jn.113.185199.

brewing are particularly rich in xanthohumol (XN)[8]. However, during the brewing process, XN isomerizes into isoxanthohumol (IX), resulting in beer having larger amounts of IX than XN (8). Up to 48 h after ingestion (9), IX is converted into 8-prenylnaringenin (8PN) in the distal colon by an *O*-demethylation catalyzed by gut microbiota (9,10).

In a recent study (11), we developed a specific methodology to analyze urinary XN, IX, and 8PN. Moreover, in an acute study with 10 volunteers who consumed a single dose of beer (330 mL), IX was the only beer prenylflavonoid able to discriminate between beer and nonbeer consumers. Accordingly, to test the potential of IX as a biomarker of beer consumption, we embarked on 3 studies: a dose-response clinical trial, an intervention clinical trial, and a cohort study of a free-living population.

Materials and Methods

Reagents and materials

Methanol (MeOH), acetonitrile, and ammonium bicarbonate of HPLC grade were used (Sigma-Aldrich). HPLC-grade acetic acid, formic acid, and ammonium acetate were purchased from Panreac Quimica. Ultrapure water (MilliQ) was generated by the Millipore System. IX, 8PN, and XN (97–99% purity) were purchased from Enzo Life Science. Taxifolin (>90% purity) was obtained from Extrasynthèse. Ascorbic acid was supplied by Sigma-Aldrich. Solid-phase extraction (SPE) cartridges Oasis MCX 96-well, 60-μm plates (30 mg) were obtained from Waters.

Identification and quantification of IX, 8PN, and XN in urine samples by SPE–liquid chromatography coupled to mass spectrometry

Prenylflavonoids in urine samples were quantified following the method previously described based on SPE and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) (11). Briefly, 1 mL of urine samples was loaded into a 96-well plate Oasis MCX cartridge previously equilibrated with 1 mL of MeOH and 1 mL of 5-mM ammonium acetate buffer adjusted to pH 5.0. Cartridges were then washed with 1 mL of MeOH:5-mM pH 5.0 ammonium acetate buffer (1:1, v/v) solution and eluted with 0.5 mL of MeOH (twice). The eluate obtained was evaporated to dryness under a gentle stream of N_2, and the residue was reconstituted with 100 μL of 5-mM pH 7.0 ammonium bicarbonate buffer containing 20 mg/L of ascorbic acid.

The identification and quantification of IX, 8PN, and XN in urine samples was performed using an HP Agilent 1100 system equipped with a quaternary pump and a refrigerated auto sampler coupled to an API 3000 triple-quadrupole mass spectrometer (AB Sciex) with a turbo ion spray source working in negative mode. Chromatographic separation was performed with a Luna C_{18} column, 50 mm $\times$ 2.0 mm i.d., 5 μm (Phenomenex), using 5-mM ammonium bicarbonate buffer adjusted to pH 7.0 as the aqueous mobile phase and acetonitrile and methanol as organic phases in a 1:1 proportion. All chromatographic conditions were previously described in detail (11). For the quantification of analytes in urine samples, the multiple reaction monitoring mode was used, monitoring 3 transitions: 353/119 (IX and XN), 339/219 (8PN), and 303/285 (taxifolin, internal standard). The MS/MS parameters are described elsewhere (11).

Taxifolin was used as the internal standard. All results were corrected for urinary creatinine, which was performed following the method adapted to the microtiter 96-well plate (6).

Analysis of IX, XN, and 8PN in beer samples by LC-MS/MS

The beer foam was removed by ultrasonication and filtered through a 0.45-μm polytetrafluoroethylene filter. Then 20 μL of beer samples were injected into the LC-MS/MS system without any other pretreatment. The identification and quantification of analytes was carried out as previously described.

Study design

The suitability of beer prenylflavonoids for use as a specific and selective biomarker of beer consumption was evaluated in 2 different intervention clinical trials (studies 1 and 2) and in a cohort study (study 3).

Study 1. A dose-response, randomized, crossover clinical trial including 41 young, healthy volunteers (20 males and 21 females, aged 28 $\pm$ 3 y, mean BMI 22.3 $\pm$ 2.6 kg/m^2) was performed. All participants drank 3 different doses of a previously selected beer (beer no. 1) the night before (2100 h), and a spot urine sample was collected in the morning (0800 h). Beer no. 1 was selected from among 19 Spanish beers based on its prenylflavonoid content (data not shown). The doses of beer administered to male volunteers were 1 beer (330 mL), 2 beers (660 mL), or 3 beers (990 mL), whereas female volunteers drank 1 beer (330 mL), 1.5 beers (495 mL), or 2 beers (660 mL). Seven days before the first intervention (run-in period), participants were asked to refrain from consuming beer, nonalcoholic beer, or hop-derived products such as hop-based food supplements. A 4-d washout period was set between interventions. After the run-in and each washout period, a blank spot urine sample was also collected in the morning. Young (21–39 y), normal weight (BMI 18.5–24.9 kg/m^2), nonsmoker participants were selected. Volunteers with a previous medical history of serious illness, alcoholism, or drug addiction, or those taking medication were excluded from the study. The different interventions were crossed and distributed randomly once per week for 3 wk.

Study 2. In a randomized, open, crossover, controlled clinical intervention trial, 33 males with high cardiovascular risk (aged 61 $\pm$ 7 y, mean BMI 29 $\pm$ 4 kg/m^2) randomly were administered 30 g of ethanol/d as gin (92 mL) or beer (660 mL of beer no. 2), or an equivalent amount of polyphenols in nonalcoholic beer (990 mL/d) for 4 wk. The participants were recruited in the outpatient clinic of the Internal Medicine Department at the Hospital Clínic of Barcelona, Spain. All were moderate alcohol consumers (1–3 drinks/d) and had diabetes or $\geq$3 of the following cardiovascular risk factors: tobacco smoking, hypertension, plasma LDL cholesterol concentrations $\geq$160 mg/dL, plasma HDL cholesterol concentrations $\leq$35 mg/dL, overweight or obesity (BMI $\geq$25 kg/m^2), and/or family history of premature coronary heart disease. Participants with documented coronary heart disease, stroke, or peripheral vascular disease, HIV infection, alcoholic liver disease, malnutrition, or neoplastic or acute infectious diseases were excluded from the study. Participants were asked not to drink alcoholic beverages 15 d before the first intervention (run-in period) and not to change their dietary pattern during the study. Diet and exercise were monitored after the run-in period and after each intervention period using a previously validated 7-d food record questionnaire (5 weekdays and 2 weekend days) (12) and the Minnesota Leisure Time Physical Activity Questionnaire, respectively. Twenty-four-hour urine collections were obtained the morning after the run-in period and after the last day of each intervention. In this study, 24-h urine collections rather than single spot urine samples were chosen to guarantee the detection of urinary prenylflavonoids, since volunteers were asked to drink the beer during the 2 main meals. All urine samples from the 2 clinical trials were coded randomly and stored at -80°C until analysis.

The beer, nonalcoholic beer, and gin were provided by the research team. Beer beverages were supplied by Centro de Información Cerveza y Salud through the Asociación de Cerveceros de España.

The study protocols of both studies were approved by the Ethics Committee of the University of Barcelona and the Institutional Review Board of Hospital Clínic, Barcelona, Spain. The volunteers were fully informed and gave written consent.

Study 3: Free-living population from the PREDIMED study cohort. To demonstrate the suitability of the new biomarker, urine samples from the free-living participants of the PREDIMED (Prevención con Dieta Mediterránea) study were analyzed. The PREDIMED is a large, parallel-group, multicenter, controlled, randomized 5-y clinical trial aimed at

[8] Abbreviations used: IX, isoxanthohumol; LC-MS/MS, liquid chromatography coupled with tandem mass spectrometry; MeOH, methanol; PREDIMED, Prevención con Dieta Mediterránea; ROC, receiver operating characteristic; SPE, solid-phase extraction; XN, xanthohumol; 8PN, 8-prenylnaringenin.

assessing the effects of the Mediterranean diet on the primary prevention of cardiovascular disease. A subsample of 46 volunteers was selected at random from the cohort studied at the Hospital Clínic to analyze urinary excretion of prenylflavonoids: 32 males and 14 females with a mean age of 63 ± 5 y and a mean BMI of 29 ± 3 kg/m^2. At baseline, participants completed a 137-item validated FFQ (13) and the validated Spanish version of the Minnesota Leisure Time Physical Activity Questionnaire (14). Five volunteers reported no beer consumption, and 41 volunteers reported drinking intermittently or daily between 22 and 825 mL/d of beer. The detailed recruitment method, study protocol, and exclusion and inclusion criteria were previously described by Estruch et al. (15,16). Spot-urine samples from all participants were collected in the morning, coded, and stored at −80°C until analysis.

The IRB of the Hospital Clínic of Barcelona approved the study protocol and each participant signed an informed consent form.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics (version 19; SPSS). Descriptive statistics (mean ± SD) were used for the baseline characteristics of the participants. Nonparametric tests were used in the statistical analysis of the data. A Wilcoxon test for related samples was performed to compare changes in response to each intervention in both clinical trials. A Mann-Whitney test for unrelated samples was used to compare responses according to gender. The female population was split into 2 according to IX excretion using the 50th percentile. A Pearson linear correlation was used to evaluate the relation between beer consumption and urinary excretion of prenylflavonoids. A receiver operating characteristic (ROC) curve was assessed to calculate the sensitivity, specificity, accuracy, and the ability to discriminate between positive and negative results of the new biomarker proposed. The cutoff point providing optimal sensitivity and specificity for the identification of beer consumers was also calculated using the ROC curve. All statistical tests were 2-tailed and the level of significance was 0.05.

Results

Analysis of beer samples

The prenylflavonoid concentrations found in the beer samples are shown in **Table 1**.

In the first study, male volunteers consumed 152 ± 23 μg, 304 ± 47 μg, and 456 ± 71 μg of IX, and 18 ± 2.4 μg, 36 ± 4.9 μg, and 55 ± 7.4 μg of 8PN in 330 mL, 660 mL, and 990 mL of beer, respectively. The intake by female volunteers was 152 ± 23 μg, 207 ± 32 μg, and 304 ± 47 μg of IX, and 18 ± 2.4 μg, 24 ± 3.3 μg, and 36 ± 4.9 μg of 8PN in 330 mL, 495 mL, and 660 mL of beer, respectively.

In the second intervention study, the daily intake of IX and 8PN was 364 ± 29 μg and 22 ± 3.5 μg, respectively, during the alcoholic beer intervention. During the nonalcoholic beer intervention, the daily intake of IX and 8PN was 184 ± 71 μg and 19 ± 2.3 μg, respectively. XN was not detected in any beer sample.

Clinical trials

Study 1. After consumption of the different volumes of beer, IX was found in the urine samples of all volunteers, whereas it was not detected after the run-in and washout periods. Urinary excretion of IX increased linearly with the dose size (**Fig. 1**) in male volunteers, showing a dose-response behavior. Regression analysis of mean IX excretion for the 3 doses in males showed a linear association (r = 0.85) (P < 0.001). However, although IX excretion in females also showed a linear relation (r = 0.89) (P < 0.001), a saturation behavior after the intake of only 1 beer (330 mL) was observed because no significant differences were observed among the 3 doses consumed. The excessively high SD in the female population showed that the females formed 2 groups based on IX excretion. Therefore, the female population was split into 2 according to IX excretion using the 50th percentile (>4.83 μg IX/g creatinine). One of the subgroups showed a dose response similar to that of the male population, whereas the other subgroup showed a saturation behavior after consuming 330 mL of beer (Fig. 1). Regression analysis including both genders at a beer dose of 0 mL, 330 mL, and 660 mL also showed a linear association (r = 0.83) (P < 0.001).

The mean urinary excretion in males was 2.4 ± 0.18, 3.3 ± 0.33, and 4.6 ± 0.45 μg/g creatinine after intake of 330 mL, 660 mL, and 990 mL of beer, respectively. In comparison, the mean IX excretion in females was higher at lower doses, 4.4 ± 0.31, 4.7 ± 0.4, and 4.9 ± 0.7 μg/g creatinine after the intake of 330 mL, 495 mL, and 660 mL of beer, respectively. Therefore, significant differences in urinary excretion of IX were observed between males and females (P < 0.001). 8PN was detected in urine samples but under the limit of quantification (<5 μg/L). XN was not detected in any urine sample from the volunteers.

Study 2. After a 4-wk intervention with alcoholic (660 mL/d) or nonalcoholic beer (990 mL/d), IX was found in all urine samples but was not detected in urine samples after the wash-out period and gin intervention. IX increased to 4.0 ± 1.6 μg/g creatinine after the beer intervention compared with the run-in and wash-out period (P < 0.001), and 4.2 ± 1.3 μg/g creatinine after nonalcoholic beer intake (P < 0.001).

PREDIMED population

In study 3, participants from the PREDIMED cohort who reported intermittent or daily beer consumption had significantly higher IX urinary concentrations than nonbeer consumers (P < 0.05). Beer consumers excreted 3.04 μg IX/g creatinine (95% CI: 1.81, 4.28μg IX/g creatinine), whereas IX was not detected in urine samples from nonbeer consumers.

ROC curves

ROC curves (Fig. 1, **Supplemental Fig. 1**) were assessed using the data obtained from the 2 clinical intervention trials and the cohort study to evaluate the effectiveness of urinary IX measurement as a biomarker of beer intake.

Using the clinical intervention trial data, the AUC was 0.990 (95% CI: 0.98, 1.00). The optimal cutoff point was 0.48 μg IX/g creatinine, which allowed differentiation between beer and nonbeer consumption with a sensitivity of 98% and specificity of 96% (likelihood ratio = 29.96, positive predictive value = 99%, negative predictive value = 96%).

Using the data from the PREDIMED study, the cutoff point of 0.48 μg IX/ g creatinine allowed discrimination between beer and nonbeer consumers with an AUC of 0.904 (95% CI: 0.797, 1.00), sensitivity of 67% (95% CI: 65, 69%), and specificity of 100%. In the free-living population, the sensitivity might be lower for different reasons: the beer dose was not established as in the clinical studies and thus beer consumption varied between 22 mL/d and 825 mL/d. In addition, both female and male volunteers were included. Despite these factors, ROC curves showed that IX is a good biomarker because the positive and negative predictive

TABLE 1 Composition of beers used in the clinical trials[1]

Beer type	IX	8PN	XN	Alcohol
	μg/L	μg/L	μg/L	%
Beer from the first intervention	460 ± 26.2	55.4 ± 0.01	nd	5.5
Beer from the second intervention	552 ± 48.8	32.6 ± 1.4	nd	5.4
Nonalcoholic beer	186 ± 7.6	19 ± 1.2	nd	0.0

[1] Values are means ± SDs unless indicated otherwise (n = 3). IX, isoxanthohumol; nd, not detected; XN, xanthohumol; 8PN, 8-prenylnaringenin.

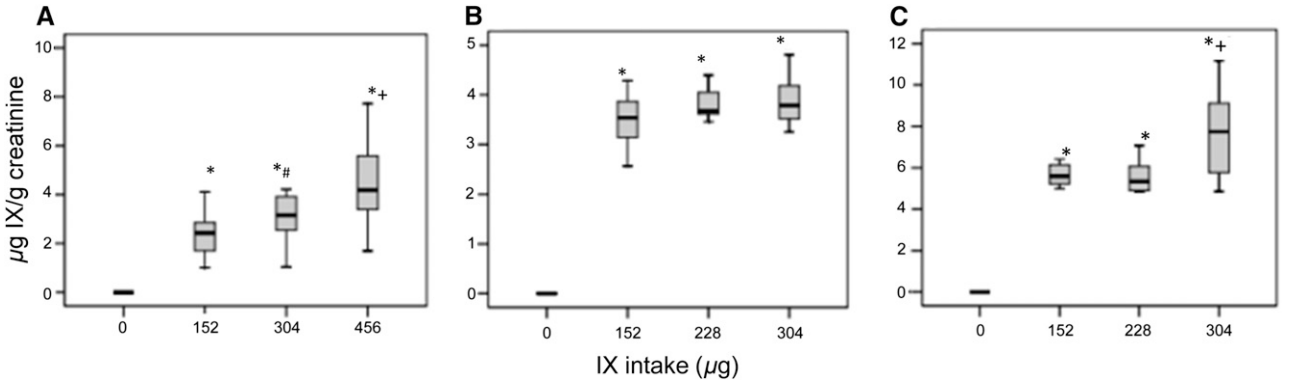

FIGURE 1 Box plot of urinary excretion of IX in males (*A*), females in the lower 50th percentile (≤4.83 μg IX/g creatinine) (*B*), and females in the upper 50th percentile (>4.83 μg IX/g creatinine) (*C*) of excretion after the intake of different doses of IX (study 1). The line in the middle of the box represents the median of the data; the bottom and top of the box are the 25th and 75th percentile, respectively. The ends of the whiskers represent the 10th and 90th percentiles [$n = 20$ (*A*), $n = 11$ (*B*), $n = 10$ (*C*)]. *Significant differences between the washout or run-in periods and the beer interventions ($P < 0.05$). #Significant differences between the first and the second IX dose according to beer intake ($P < 0.001$). +Significant differences between the second and the third IX dose according to beer intake ($P < 0.05$). IX, isoxanthohumol.

values were still high, 70% and 100%, respectively. Therefore, according to the traditional academic point system, the accuracy of the new biomarker was found to be excellent (AUC > 0.9).

Discussion

The development of potent new nutritional biomarkers is crucial for epidemiologic research because the more objective data they provide allow a more accurate assessment of food and nutrient intake of the participants evaluated. Nutritional biomarkers are also very useful in clinical trials for monitoring compliance with administered interventions. New biomarkers need to be validated in 2 stages: *1*) in dose-response, controlled clinical trials to identify the range of intake in which the biomarker is reliable, and *2*) in free-living populations to evaluate the suitability of the biomarker in a habitual diet (17). Moreover, an effective biomarker of food intake should be specific to the dietary component of interest, sensitively reflect changes in food intake by its concentration in the biofluid, have an adequate half-life in the biofluid, provide good correlation between excretion and exposure, and have a robust quantification method (18,19). Taken as a whole, the results of the current studies suggest that urinary IX excretion is an excellent biomarker of alcoholic and nonalcoholic beer intake because it fulfills all the aforementioned criteria. Unlike other polyphenol-based biomarkers (4–6), IX is specific to only 1 dietary component (beer), being found exclusively in hops, which makes it an ideal and highly sensitive biomarker of beer intake. In fact, a cutoff point of 0.48 μg IX/g creatinine allowed perfect discrimination between beer and nonbeer drinkers. AUCs obtained in both ROC curves, using the data from the clinical trials and PREDIMED cohort, were >0.9, which means that IX is a highly accurate biomarker of beer intake according to the traditional academic point system. Furthermore, IX proved to be a good biomarker of both alcoholic and nonalcoholic beer because no differences in IX urinary excretion were found between the intervention periods of both types of beer, even though the regular beer had a higher concentration of prenylflavonoids.

In this study, urinary IX excretion was also investigated in a dose-response clinical trial. Interestingly, IX recovery in urine samples increased linearly with dose size in male volunteers, whereas a saturation behavior was observed after a single dose of 1 beer (330 mL) in 1 subgroup of the female population. Gender differences in urinary polyphenol recovery have been reported previously for isoflavones (20,21) and may be due to differences in absorption and metabolism. In general, female volunteers excreted twice as much IX after the consumption of 1 beer (330 mL) as male volunteers, yet in a subgroup of female volunteers, no differences in IX excretion were found after 1.5 and 2 beers, suggesting that in some women IX excretion may be saturated at 152 μg. In contrast, male and some of the female volunteers excreted IX linearly, depending on the size of the beer dose. Intestinal absorption of IX has not been previously studied, but if IX behaves similarly to XN, then an active efflux pump system is expected to be responsible for the uptake of IX (22). XN has shown high permeability in Caco-2 cells, and once inside the cell, 90% of XN remains trapped in the cytosol bound to a cytosolic protein in a process that is temperature-dependent and saturable (22). Therefore, a saturation of IX absorption, similar to XN, could be expected, and our results suggest the dose of saturation differs between genders.

In summary, urinary IX was validated as a biomarker of beer consumption. Measurement of urinary IX concentration proved to be a specific and accurate biomarker of beer intake, which might be expected because it is a component exclusive to beer in a regular diet. This new biomarker can be used to discriminate between nonbeer and beer consumers, and it may be a useful tool for monitoring participant compliance in intervention trials. However, IX might be less effective in distinguishing intermittent female beer consumers because IX urinary excretion in some women is not dose-dependent.

Acknowledgments

R.M.L.-R. and R.E. designed the research; P.Q.-R., M.M.-H., and G.C.-B. conducted the research; O.J. provided technical support; P.Q.-R. analyzed the data; and P.Q.-R., G.C.-B., R.E., and R.M.L.-R. wrote the paper. P.Q.-R. and G.C.-B. thank the Centro de Información Cerveza, Salud y Nutrición for the Manuel de Oya fellowship. M.M.-H. thanks the predoctoral program of MICINN. The authors thank the Asociación de Cerveceros de España for providing the nonalcoholic and regular beers, and Gin Xoriguer for providing the gin used in this study. All authors read and approved the final manuscript.

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4.2.4. Publication 5. Non-alcoholic beer fraction induces changes in human fatty oxidation and amino acid metabolism: a metabolomics approach.

Paola Quifer-Rada, Gemma Chiva-Blanch, Olga Jáuregui, Ramon Estruch, Rosa M. Lamuela-Raventos. *Molecular Nutrition and Food Research*, submitted.

Summary:

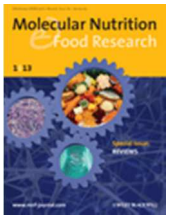
Moderate alcohol consumption has been inversely associated with incidence of cardiovascular risk factors and all-cause mortality in several studies, independently of the type of alcoholic beverage consumed. However, the results of some studies indicate that fermented alcoholic beverages, like wine or beer, may provide additional protective effects due to their polyphenolic content. The aim of this work was to study the urinary metabolomics changes of participants that consumed beer, non-alcoholic beer and gin in order to evaluate metabolic shifts related to the beverage type and discovery of new potential biomarkers of beer consumption.

Thirty-three males at high cardiovascular risk between 55 and 75 years old participated in an open, randomized, crossover, controlled trial with three nutritional interventions consisting of beer (660 mL per day), non-alcoholic beer (990 mL/day) and gin (100 mL/day), each administered for four weeks in a random sequence. Diet and physical activity was monitored throughout the study and compliance was assessed by measurement of urinary isoxanthohumol, a biomarker of beer and non-alcoholic beer intake.

Metabolomic analysis was performed in urine samples by liquid chromatography coupled to an LTQ-Orbitrap mass spectrometer combined with univariate and multivariate statistical analysis to identify metabolomic changes after each intervention.

A total of 10 metabolites were identified discriminating between interventions and baseline point. After the beer and non-alcoholic beer interventions, we observed increased urine excretion of hop alpha acids like humulinone, cohumulone and oxyhumilinic acid, as well as 2,3-dihydroxy-3-methylvaleric acid, which is a fatty acyl formed during fermentation, and 1,2,3,4-tetrahydro-1-methyl-β-carboline-3-carboxylic acid, which is an aromatic heteropolycyclic compound normally found in alcoholic fermented beverages. After the gin and beer interventions, metabolites from the alcohol detoxification process like ethyl sulfate, 2-phenylethanol glucuronide, and ethyl glucuronide increased in urine. Moreover, beer and non-alcoholic beer intake increase the excretion of two endogenous metabolites: hydroxyadipic acid linked to fatty acid oxidation, and 4-guanidinobutanoic acid involved in arginine metabolism, which was also correlated with a decrease in urinary creatinine.

In conclusion, Beer and non-alcoholic beer changed the urine metabolic profile. The results of this trial provide new evidence that the non-alcoholic fraction of beer may increase fatty oxidation and increase amino acid metabolism. Moreover, humulinone is a potential new biomarker of beer consumption, although a full validation of the biomarker, as performed for isoxanthohumol, is needed.



Non-alcoholic beer fraction induces changes in human fatty oxidation and amino acid metabolism: a metabolomics approach

Journal:	*Molecular Nutrition and Food Research*
Manuscript ID	mnfr.201600980
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	08-Nov-2016
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Keywords:	Alcohol, Gin, Beer, polyphenols, metabolomics, urine metabolome, fatty acid oxidation

Research Article

Non-alcoholic beer fraction induces changes in human fatty oxidation and amino acid metabolism: a metabolomics approach

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Keywords: Alcohol, gin, beer, polyphenols, metabolomics, urine metabolome, fatty acid oxidation

Abbreviations: CVD, cardiovascular diseases; **LC-HRMS,** liquid chromatography coupled to high resolution mass spectrometry; **MeCN,** acetonitrile; **MeOH,** Methanol; **na-beer**, Non-alcoholic beer; **PCA,** Principal Component Analysis; **PLS-DA,** Partial Least Square Discriminant Analysis; **QC,** Quality controls; **VIP,** Variable Important for the Projection.

Abstract

Scope: The aim of this study was to perform a metabolomic analysis of urine of participants that consumed beer, non-alcoholic beer and gin in order to evaluate metabolic changes related to the beverage type.

Methods and results: 33 males between 55 and 75 years old participated in an open, randomized, crossover, controlled trial with three nutritional interventions consisting of beer (660 mL per day), non-alcoholic beer (990 mL/day) and gin (100 mL/day), each administered for four weeks in a random sequence. Metabolomic analysis was performed in urine samples by liquid chromatography coupled to an LTQ-Orbitrap mass spectrometer combined with univariate and multivariate statistical analysis to identify metabolomic changes after each intervention.

A total of 10 metabolites were identified. Eight were exogenous metabolites related to beer, non-alcoholic beer or gin consumption, but two of them were related to metabolic endogenic changes: hydroxyadipic acid linked to fatty acid oxidation, and 4-guanidinobutanoicacid involved in arginine metabolism, which was correlated with a decrease in urinary creatinine.

Conclusion: Beer and non-alcoholic beer changed the urine metabolic profile. The results of this trial provide new evidence that the non-alcoholic fraction of beer may increase fatty oxidation and increase amino acid metabolism.

1 Introduction

Moderate alcohol consumption has been inversely associated with incidence of cardiovascular risk factors and all-cause mortality in several studies, independently of the type of alcoholic beverage consumed [1–3]. However, the results of some studies indicate that fermented alcoholic beverages, like wine or beer, may provide additional protective effects due to their polyphenolic content [4–6].

In a previous clinical trials we evaluated the protective effects of beer, non-alcoholic beer (na-beer) and gin in 33 men at high cardiovascular risk [7, 8]. We reported that moderate daily consumption of beer and non-alcoholic beer exerts higher protective effects on cardiovascular health than distilled beverages such as gin [5, 8]. Moderate consumption of beer or na-beer, but not gin, significantly decreased cellular and plasma biomarkers related to atherosclerosis, such as lymphocyte expression of lymphocyte function-associated antigen1 and SLe^x (Sialil-Lewis X), monocyte expression of SLe^x and chemokine receptor type 2, and increased interleukine (IL)-1-ra (interleukin-1 receptor agonist). Moreover, daily consumption of na-beer also decreased systolic blood pressure and significantly decreased IL-6, IL-15, regulated on activation, normal T-cell expressed and secreted and tumor necrosis factor beta [7], whereas moderate consumption of alcohol (as beer or gin) improved the lipid profile by significantly increasing HDL-cholesterol, Apo A-I, Apo A-II and adiponectin [7]. Furthermore, moderate consumption of both beer and na-beer, but not gin, increased the number of circulating endothelial progenitor cells in peripheral blood [8], which have been proposed as a marker for vascular function and cumulative cardiovascular risk [9].

The aim of metabolomics is to comprehensively analyze all metabolites present in biological systems. This novel approach has been used in the field of nutritional science to study the influence of dietary compounds on the metabolic profile of biological fluids [10–14]. In this work, liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS) has been the analytical technique of choice. LC-HRMS-based metabolomics usually implies the use of a time-of-flight (ToF) mass analyzer; however, other HRMS detectors, such as Orbitrap,

can be used. Glauser et al. compared Orbitrap and ToF-MS performances and concluded that both technologies provided very similar results regarding the detected markers and sample classification, and both platforms were equally efficient for untargeted metabolomics [15]. In this setting, we used an LC-HRMS Orbitrap to perform a metabolomic analysis of urine of 33 high cardiovascular risk subjects in order to evaluate possible metabolic changes after the daily moderate consumption of beer, na-beer or gin.

2 Materials and Methods

2.1 Chemicals and reagents

Kaempferol-3-*O*-glucoside, hesperidin, 3-hydroxybenzoic acid, p-coumaric acid, caffeic acid, gentistic acid, homovanillic, naringenin, naringenin-7-*O*-glucoside and hippuric acid (all 97-99% purity) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol (MeOH) and acetonitrile (MeCN) of HPLC grade were obtained from Sigma-Aldrich (St. Louis, MO, USA). HPLC formic acid was purchased from Panreac Quimica S.A (Barcelona, Spain). Ultrapure water (MilliQ) was generated by the Millipore System (Bedford, MA, USA).

2.2 Study design and subjects

The study protocol has been reported in previously published papers [7, 8]. In brief, 33 males between 55 and 75 years old completed an open, randomized, controlled, crossover trial with three nutritional interventions. The subjects were moderate alcohol drinkers (1-3-drinks/day) at high cardiovascular risk (diabetics or with three or more cardiovascular risk factors: family history of premature cardiovascular disease (CVD) and/or presence of hypertension, dyslipidemia, and overweight/obesity). Exclusion criteria included documented CVD, human immunodeficiency virus infection, chronic liver disease, malnutrition, neoplastic or acute infectious diseases and regular use of vitamin supplements.

Participants underwent the three interventions in a randomized sequence in order to counteract a possible carryover effect. Participants were instructed to consume beer (660 mL per day,

containing 30 g of alcohol and 1029 mg of polyphenols), non-alcoholic beer (990 mL/day, containing <1g of alcohol and 1243 mg of polyphenols) and gin (100 mL/day, containing 30 g of ethanol and no polyphenols) at dinner, with each intervention lasting four weeks. The beverages were provided to the participants, and the beer and non-alcoholic beer used in the study were a type of lager from the same Spanish commercial brand. In each intervention, subjects were asked to abstain from other alcoholic beverages, except for those provided by the investigators. Two weeks prior to the study, subjects were asked to follow their usual diet but to refrain from consuming any alcoholic beverage (run-in period). There was no wash-out period between interventions. Baseline data were collected after the run-in period. Samples of fasting blood and 24-h urine were collected after the run-in period and at the end of each intervention, and stored at -80ºC.

Diet and physical activity was monitored throughout the study, as explained previously [7, 8]. Compliance was assessed by measurement of urinary isoxanthohumol, a biomarker of beer and non-alcoholic beer intake [16].

The Institutional Review Board of the Hospital Clinic approved the study protocol and all participants gave written consent. The trial was registered in the Current Controlled Trials at London (http://www.controlled-trials.com), International standards Randomized Controlled Trial Number at http://www.isrctn.org as ISRCTN95345245.

2.3 Sample preparation

Aliquots of 24-h urine were centrifuged at 12,000 g for 5 minutes. Then 500 µL of urine was diluted with 500 µL of Milli-Q water, filtered through a 0.22 µm filter and transferred to an amber HPLC vial for injection.

2.4 Liquid chromatography-high-resolution mass spectrometry

The liquid chromatography coupled to an LTQ-Orbitrap method has been previously reported [17]. Briefly, HPLC analysis was performed on an Accela chromatograph (Thermo Scientific,

Hemel Hempstead, UK) equipped with a quaternary pump and a thermostated autosampler. Urine metabolites were eluted using an Atlantis T3 column 2.1x100 mm, 3µm (Waters). Water with 0.1% formic acid and acetonitrile with 0.1% formic acid were used as aqueous (A) and organic (B) mobile phases, respectively. Gradient elution was: 0 min, 2% B; 2 min, 2% B; 5 min, 8% B; 14 min, 20% B; 18 min, 30% B; 22 min, 100% B; 24 min, 100% B; 25 min, 2% B; 30 min, 2% B. Flow rate was set at 350 µL/min and the injection volume was 10 µL.

An LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Hemel Hempstead, UK) equipped with an ESI source working in negative and positive mode was used for accurate mass measurements. Mass spectra were acquired in profile mode with a setting of 30,000 resolution at *m/z* 400. Operation parameters have been previously described in detail [17, 18]. Urine samples were analyzed in full-scan mode and data-dependent MS/MS events were acquired at a resolving power of 15,000. The mass range was from m/z 100 to 1000. Data-dependent scanning was carried out without the use of a parent ion list. Ions that were not intense enough for a data-dependent scan were analyzed in MS^2 mode with the Orbitrap. Precursor ions were fragmented by collision-induced dissociation C-trap (CID) with normalized collision energy of 35 V and an activation time of 10 ms. High-resolution MS data monitoring and calculation of elemental composition was performed using XCalibur software v2.0.7 (Thermo Fisher Scientific). An external calibration for mass accuracy was carried out before the analysis.

2.5 Quality controls

Quality controls (QC) were prepared by pooling ten phenolic compounds in the mobile phase, including kaempferol-3-*O*-glucoside, hesperidin, 3-hydroxybenzoic acid, p-coumaric acid, caffeic acid, gentistic acid, homovanillic, naringenin, naringenin-3-*O*-glucoside and hippuric acid. QC were injected randomly during the sequence and once the sequence was finished, in order to control retention time shifts and mass accuracy. The accepted criteria of QC were: a maximum variance of 0.15 min of retention times and a mass accuracy of 3 ppm.

2.6 Data management and statistical analysis

Feature detection, peak alignment and retention time correction were performed using the XCMS-R package [19, 20] and the following parameters were set: mass tolerance 2.5 ppm, peak width range 10-60 seconds, 3 minimum scans, minimum intensity of 5000 arbitrary units. The method used for peak detection was "centWave", and for retention time correction "obiwarp" with a step size of 1 *m/z*. Peak grouping was performed using a width of overlapping m/z slices of 0.015, a minimum fraction of samples for grouping of 0.5 and a bandwidth of 5. The preprocessed data obtained by XCMS in positive and negative ionization was further filtered by sample representativeness using the 80% rule and by intensity criteria and coefficient of variation. The 80% rule consists of retaining those mzRT features that are consistently found in 80% of the samples or at least in one experimental group. In the coefficient of variation criteria filtration, a variation of 20% was the minimum accepted variation across samples to consider a biological variation. Additionally, the intensity threshold was set at 30,000 arbitrary units as the minimum suitable intensity for MS/MS experiments in the intensity criteria. Univariate analysis was performed with R. T-test analysis was used to compare the "control" (baseline) with "beer", "na-beer" (non-alcoholic beer) and "gin" groups. The false discovery rate was applied to correct false positive results and a significance level of 0.05 was set. Unsupervised principal component analysis (PCA) was used to check the quality of the data acquisition, including the four classes of control, beer, na-beer and gin.

Also, filtered data were exported to SIMCA 13.0.3 software (Umetrics) to perform multivariate analysis. The supervised model of partial least square discriminant analysis (PLS-DA) was used to analyze urine metabolomic differences between the four interventions. Four PLS-DA analyses were conducted: three pairwise analyses to compare control versus beer, na-beer and gin intervention, respectively, and one to compare the four classes together. The data sets were normalized and Pareto-scaled before PCA and PLS-DA analysis.

The quality of the model was evaluated using the goodness-of-fit parameter (R^2X), the proportion of the variance of the response variable explained by the model (R^2Y), and the predictive ability parameter (Q^2).

Metabolites discriminating between the beer, na-beer and gin samples versus control samples (VIP> 1), and features showing an adjusted p-value<0.05 and a fold change (FC)>1.5 were putatively identified by exact mass matching using the METLIN Metabolite, Human Metabolome, Lipid MAPS and Mass Bank databases[21–25] with a mass accuracy of 5 ppm. Metabolites were further confirmed by MS/MS experiments and comparing the spectra with data from the databases and the literature. The molecular formula was calculated using the exact mass and further confirmed by matching with the isotopic pattern.

2.7 Creatinine determination

Creatinine analysis was performed using the Jaffé alkaline picrate method [26] adapted to 96-well microtiter plates [27]. Briefly, 3 µL of urine was reacted with 60 µL of aqueous picric acid solution (1%) and 5 µL of sodium hydroxide (10%). After 15 minutes in darkness at room temperature, 232 µL of Milli-Q water was added. The absorbance was measured at 500 nm by a UV/VIS spectrophotometer. To quantify creatinine content in urine samples, calibration curves were prepared with a creatinine standard. Changes in creatinine excretion among interventions were evaluated by the Mann Whitney U paired test using R software.

3 Results

3.1 Data processing

After processing the data with XCMS, 175,104 and 56,584 features in negative and positive, respectively, were detected in the 30-minute chromatogram (acquired m/z range of 100-1000). After filtering for sample representativeness (the 80% rule), 55.3% and 63.9% of the features in negative and positive, respectively, were retained. The data were further filtered by intensity and quality control criteria. After filtering for intensity criteria, 83.9% and 54.8% of the features

in negative and positive, respectively, were retained. Features showing a lower intensity than 30,000 arbitrary units and a CV<20% were removed, and 83.1% and 52.3% of the mzRT features in negative and positive, respectively, fulfilled the criteria.

After filtering the data, a t-test adjusted by FDR was performed and the fold change (FC) of the mean peak intensity between "control" versus "beer", "na-beer" and "gin" samples were calculated. Only 168 and 27 features in negative and positive, respectively, showed an adjusted p-value<0.05 and a FC>1.5.

3.2 Quality control

Quality controls were performed in all mass spectrometry analyses to evaluate the quality of the acquisition. Intra- and interbatch retention time shifts were lower than 0.1 min, peak area variation was lower than 18% and mean mass accuracy was 2 ppm. Moreover, principal component analysis of the quality control injections was also plotted to dismiss clustering among batches.

3.3 Multivariate analysis

The resulting PLS-DA model including the four classes (control, beer, na-beer and gin) was useful to visualize clustering and distribution of the classes, but the predictive ability of the model was weak and not significant ($Q^2 \leq 0.47$) (**Table 1 and Figure 1**). Therefore, we used pairwise PLS-DA models of control vs. beer, control vs. na-beer, and control vs. gin (**Figure 1**). Compared to the control models, beer and na-beer showed sufficient discrimination between classes, with robustness and predictability, to explain the differences between the interventions ($R^2X \geq 0.66$, $R^2Y \geq 0.74$, $Q^2 \geq 0.62$) (**Table 1**). The gin vs. control PLS-DA model did not show enough discriminatory power ($R^2X \leq 0.55$, $R^2Y \leq 0.51$, $Q^2 \leq 0.44$) between the two sample types, suggesting that overall the urine metabolome did not significantly change during the gin intervention compared to baseline, at least according to this methodology.

3.4 Metabolite identification

Features that were significant in the univariate analysis and/or in the discriminant analysis were putatively identified (**Table 2**). A total of 10 metabolites related to beer, na-beer or gin consumption were identified. One metabolite was associated with fatty acid oxidation, another was related to arginine metabolism, and 8 metabolites were exogenous. Three of the latter were related to the hop polyphenol fraction in beer and na-beer, 3 to alcohol metabolism, and 2 are found in beer: a fatty acyl and an aromatic heteropolycyclic compound.

All metabolites were confirmed by MS/MS spectra and comparing the fragments found with the literature [18, 28–34] and databases such as METLIN, Human Metabolome Database and Mass Bank.

3.5 Creatinine

Figure 2 shows the results of the creatinine determination. Urinary creatinine excretion significantly decreased after the beer and na-beer interventions compared to baseline, but did not change significantly during the gin intervention. These results are in concordance with the increased excretion of 4-guanidinobutanoic acid after beer intervention, identified by the PLS-DA and univariate analysis using the data of the untargeted metabolomics.

4. Discussion

This study aimed to evaluate the metabolic shifts that occurred after four weeks of daily consumption of beer, na-beer or gin (a polyphenol-free alcoholic beverage). We observed that beer and na-beer consumption significantly changed the urinary metabolome according to the PLS-DA models, whereas changes after gin consumption were not significant. Thus, hydroxyadipic acid excretion increased around 3- and 2.5-fold after the beer and na-beer intervention, respectively (**Table 2**). Hydroxyadipic acid is a dicarboxylic acid formed by fatty acid ω-oxidation. Dicarboxylic acid excretion increases under conditions of high fatty acid oxidation or starvation. Mitochondria and peroxisomes are responsible for the β-oxidation of

lipids, but peroxisomes also oxidize steroids and specific fatty acids such as very long chain fatty acids, branched fatty acids, bile acids and dicarboxylic acids. Peroxisomal β-oxidation of dicarboxolic acids produces medium-chain dicarboxylic acids like apidic acid (C6) and suberic acid (C8) [35]. PPARs (peroxisomal proliferator activated receptors) are nuclear receptors that play a key role in regulating energy metabolism. It has been shown that PPARα regulates the adaptive fasting response by increasing mitochondrial β-oxidation, ketogenesis and microsomal ω-oxidation. In turn, polyphenols have been shown to modulate PPAR activity. Catechin, genistein and citrus polymethoxylated flavones activate PPARα [36–38], and polyphenols from lemon up-regulate mRNA levels of PPARα, activating the nuclear receptor [39]. *p*-Coumaric acid increases the mRNA expression of PPARα, indicating that it may activate fatty acid oxidation in skeletal muscle cells, and naringenin increases fatty acid oxidation by up-regulating the gene expression of enzymes implicated in peroxisomal β-oxidation [40]. Additonally, isohumulones, the hop bitter acids in beer, reduce insulin resistance and plasma lipid concentration by activating PPARα in diabetic mice [41]. Therefore, we hypothesized that beer polyphenols and isohumulones may activate PPARα and increase liver fatty acid β- and ω-oxidation, resulting in an enhanced excretion of urinary hydroxyapidic acid.

Urinary excretion of 4-guanidinobutanoic acid increased 1.63-fold after the beer and na-beer interventions. 4-Guanidinobutanoic acid is a common urinary metabolite arising from the metabolism of arginine and proline (creatinine pathway) and is also related to nitric oxide (NO) production. In accordance with our findings, Yokozawa et al. 2003 [42] showed that green tea polyphenols increase urinary excretion of guanidino compounds and significantly decrease creatinine excretion in rats. It is well known that polyphenols increase endothelial synthesis of NO and cause NO-mediated endothelium-dependent relaxation, reducing blood pressure [43, 44]. In a previous study, we observed that systolic blood pressure decreased by a mean of 4 mmHg during a na-beer intervention, suggesting that the non-alcoholic fraction of beer, composed mainly of polyphenols, reduces blood pressure, and since blood pressure did not change after consumption of alcoholic beer, ethanol seems to act as an antagonist of the

hypotensive effect of non-alcoholic compounds of beer. However, plasmatic NO remained constant in all interventions [7]. Therefore, beer polyphenols may increase the metabolism of arginine, and slightly increase the excretion of guanidino compounds, but this pathway may not be sufficiently up-regulated by polyphenols to observe increased levels of plasmatic NO.

In addition, several exogenous metabolites from the beer or gin matrix were identified in the urine samples. After the beer and na-beer interventions, we observed increased urine excretion of hop alpha acids like humulinone, cohumulone and oxyhumilinic acid, as well as 2,3-dihydroxy-3-methylvaleric acid, which is a fatty acyl formed during fermentation [45], and 1,2,3,4-tetrahydro-1-methyl-β-carboline-3-carboxylic acid, which is an aromatic heteropolycyclic compound normally found in alcoholic fermented beverages. After the gin and beer interventions, metabolites from the alcohol detoxification process like ethyl sulfate, 2-phenylethanol glucuronide, and ethyl glucuronide increased in urine.

The hop alpha acids identified have potential use as biomarkers of beer consumption. However, in order to propose a new biomarker of exposition, a VIP value from the PLS-DA analysis higher than 1 is necessary and only humulinone fulfilled this criterion. Humulinone is an oxidized product of humulone normally found in beer [29]. In a previous study, we validated isoxanthohumol (a prenylflavanoid from hops) as a biomarker of beer consumption using two control clinical trials and a cohort study [16]. However, the analytical conditions to quantify prenylflavanoids are very specific, since they are unstable in acid medium, as we reported [46], and the LC conditions used in that work were not adequate for their identification. Therefore, humulinone is a potential new biomarker of beer consumption, although requiring validation, as performed for isoxanthohumol.

In conclusion, beer and non-alcoholic beer changed the exogenous and endogenous urine metabolic profile. This work provides new evidence that the non-alcoholic fraction of beer may increase fatty oxidation and increase amino acid metabolism. However, more targeted studies evaluating these particular effects are needed to confirm these hypotheses.

Author contributions:

PQR have carried out all metabolomics analysis and written the manuscript, GCB have performed recruitment and all data collection, RE, RMLR and GCB have designed the study, and RE, RMLR and OJ have supervised the study. All authors have read and approved the final version of the manuscript

Acknowledgements:

Paola Quifer is grateful for the predoctoral fellowships awarded by the Generalitat de Catalunya (FI-DRG).

We thank the Asociación de Cerveceros de España for providing the non-alcoholic and regular beers, and Gin Xoriguer for providing the gin used in the study.

This work was supported in part by ERAB EA 1117, EA 1324 and EA 1517, CICYT (AGL2013-49083-C3-1-R), the Instituto de Salud Carlos III, ISCIII (CIBEROBN) from the Spanish Ministry of Economy and Competivity (MEC) and Generalitat de Catalunya (GC) 2014 SGR 773.

Conflict of interest:

Dr. Lamuela-Raventos reports serving on the board of and receiving lecture fees from FIVIN; receiving lecture fees from Cerveceros de España; and receiving lecture fees and travel support from PepsiCo. Dr. Estruch reports serving on the board of and receiving lecture fees from the Research Foundation on Wine and Nutrition (FIVIN); serving on the boards of the Mediterranean Diet Foundation, the Beer and Health Foundation and the European Foundation for Alcohol Research (ERAB); receiving lecture fees from Cerveceros de España and Sanofi-Aventis; and receiving grant support through his institution from Novartis. Nevertheless, these foundations were not involved in the study design, the collection, analysis and interpretation of data, the writing of the manuscript or the decision to submit the manuscript for publication.

The other authors declare no conflict of interest.

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Tables

Table 1. Summary statistics of PLS-DA modeling quality.

Table 2. List of metabolites tentatively identified by discriminating between baseline and 4-weeks of each intervention (Beer, na-Beer and Gin).

Figure legends

Figure 1. PLS-DA score plots of urine samples.

Figure 2. Urinary creatinine excretion at baseline and after 4-weeks of each intervention. *Significantly different from before the intervention (Mann Whitney U paired test).

Table 1. Summary statistics of PLS-DA modeling quality

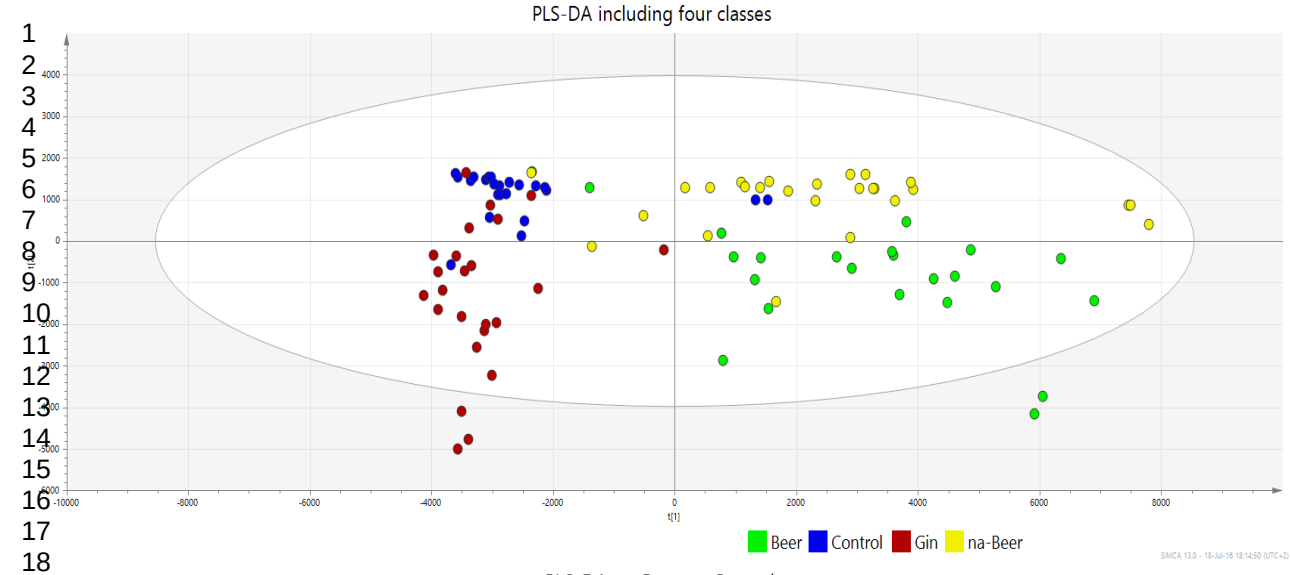
PLS-DA model	Polarity	R^2X (cum)[a]	R^2Y (cum)[a]	Q^2 (cum)[b]
Four classes	Negative	0.71	0.52	0.47
	Positive	0.75	0.33	0.28
Control *vs* Beer	Negative	0.67	0.79	0.72
	Positive	0.80	0.83	0.77
Control *vs* na-Beer	Negative	0.66	0.74	0.62
	Positive	0.74	0.81	0.71
Control *vs* Gin	Negative	0.29	0.51	0.44
	Positive	0.55	0.41	0.21

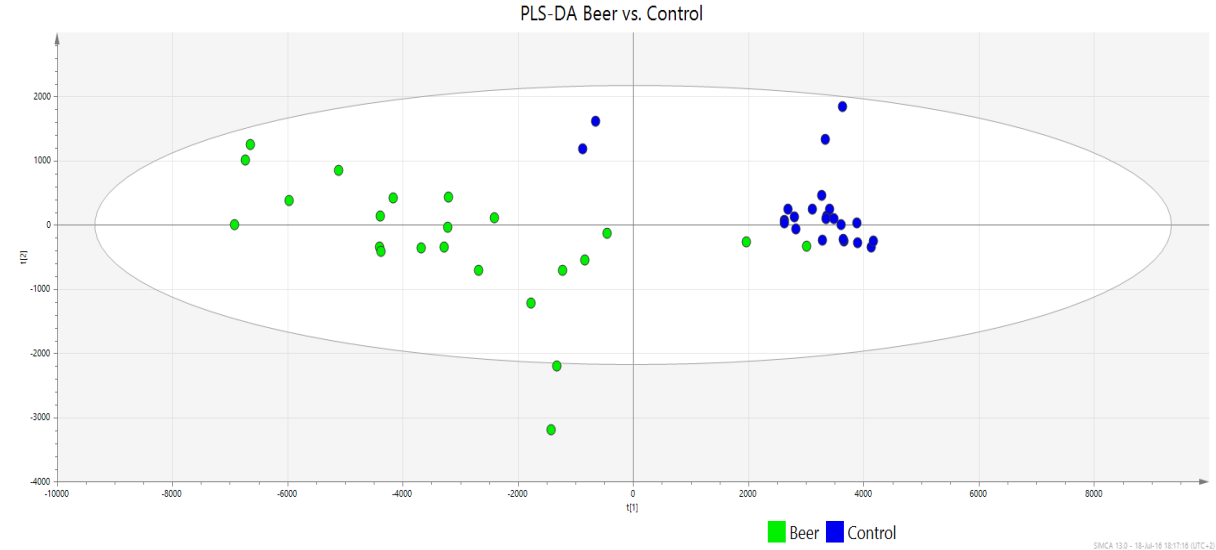
[a] R^2X (cum) and R^2Y (cum) are the cumulative explained variation in X and Y, respectively.
[b] Q^2 (cum) is the predicted variation.

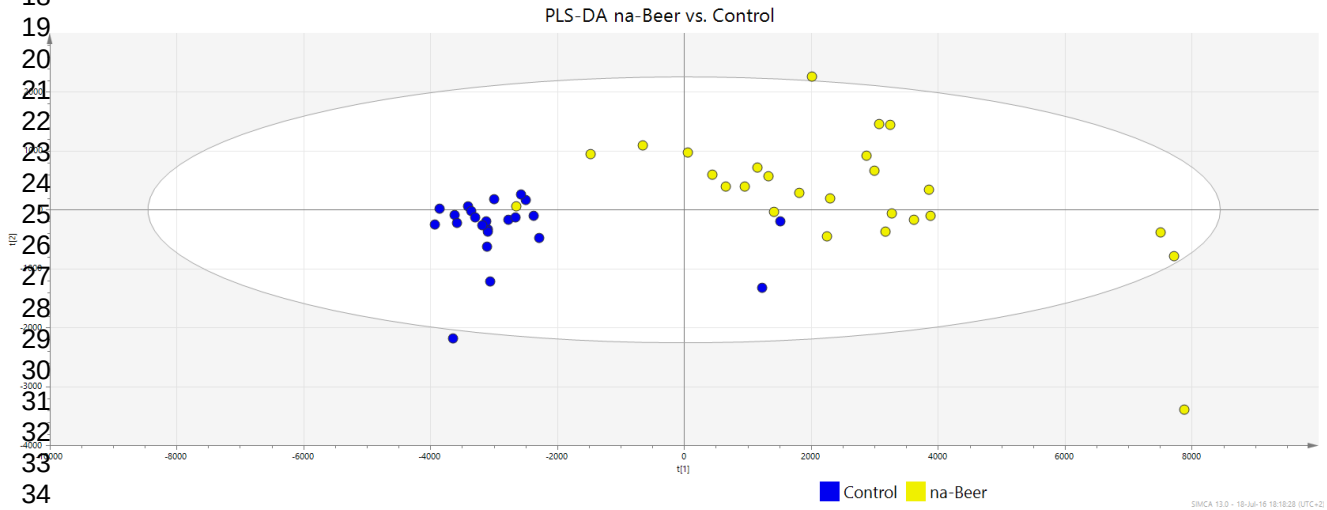
Table 2. List of metabolites tentatively identified discriminating between baseline and 4-weeks of each intervention (Beer, na-Beer and Gin).

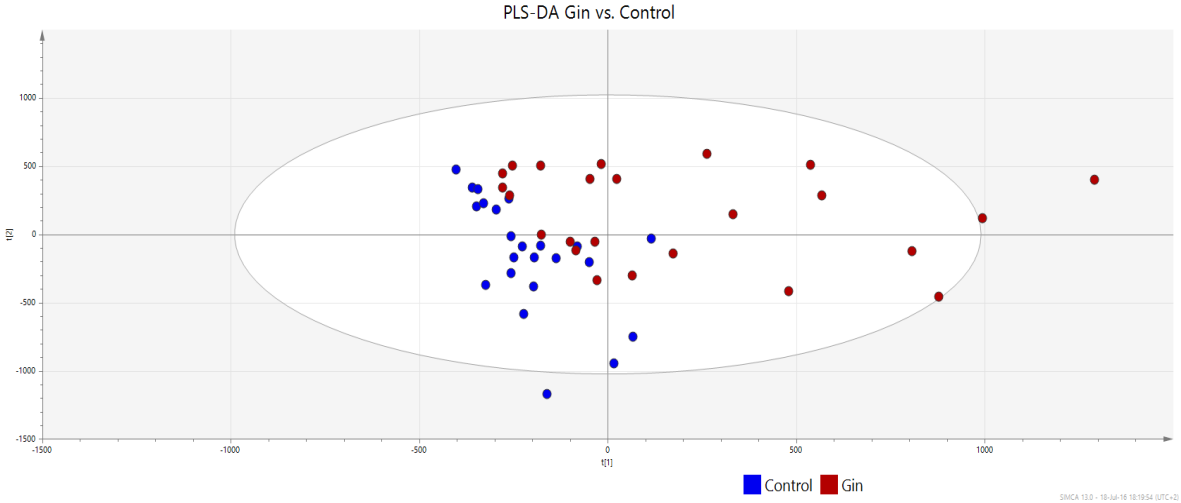
Metabolite	Metabolic Pathway	Molecular formula	*m/z* detected	Error (ppm)	Fold Change	*p*-value[a]	VIP	MS^2 Fragments	Model
Ethyl sulfate	Alcohol metabolism	$C_2H_5O_4S$	$[M-H]^-$ 124.9912	1.6	7.8	<0.001	6.6	96.9599	Control vs. Beer
					8.4	<0.001	-		Control vs. Gin
2-Phenylethanol glucuronide	Alcohol metabolism	$C_{14}H_{17}O_7$	$[M-H]^-$ 297.0975	1.6	35.7	<0.001	5.8	279.0870, 113.0242, 217.0502, 193.0348, 175.0244, 157.0140	Control vs. Beer
Hydroxyadipic acid	Fatty acid oxidation	$C_6H_9O_5$	$[M-H]^-$ 161.0453	2.0	3	<0.01	5.2	279.0870, 113.0242, 217.0502, 193.0348, 175.0244, 157.0140	Control vs. Beer
					2.4	0.2	5.2		Control vs. na-Beer
Ethyl glucuronide	Alcohol metabolism	$C_8H_{13}O_7$	$[M-H]^-$ 221.0664	1.5	26.4	<0.01	5.6	203.0557, 113.0242, 129.0191, 85.0293, 75.0086	Control vs. Beer
					33.1	<0.001	-		Control vs. Gin
Humulinone	Hop polyphenols	$C_{21}H_{29}O_6$	$[M-H]^-$ 377.1964	1.5	5.7	<0.001	4.2	281.1387, 265.1437,251.1281,195.0657,235.1333,223.0604	Control vs. Beer
					4	0.05	4.2		Control vs. na-Beer
Oxyhumulinic acid	Hop polyphenols	$C_{15}H_{21}O_5$	$[M-H]^-$ 281.139	1.5	8.3	<0.001	0.82	263.128, 155.0709, 179.0710	Control vs. Beer
					6.5	<0.01	0.83		Control vs. na-Beer
Cohumulone	Hop polyphenols	$C_{20}H_{28}O_5$	$[M-H]^-$ 347.1857	2.4	6.9	<0.001	0.61	251.1280, 235.1332, 278.1150, 265.1436	Control vs. Beer
					6.9	<0.01	0.77		Control vs. na-Beer
2,3-Dihydroxy-3-methylvaleric acid	Beer fatty acyl	$C_6H_{12}O_4$	$[M-H]^-$ 147.066	1.9	2.2	<0.001	2.76	75.0085, 129.0189, 85.0292,101.0604,71.0500	Control vs. Beer
4-Guanidinobutanoic acid	Arginine metabolism	$C_5H_{11}N_3O_2$	$[M+H]^+$ 146.0926	1.5	2.2	0.04	1.63	128.0826,104.0711, 87.0444	Control vs. Beer
					2.1	0.2	1.63		Control vs. na-Beer
1,2,3,4-Tetrahydro-1-methyl-β-carboline-3-carboxylic acid	Beer aromatic heteropolycyclic compound	$C_{13}H_{14}N_2O_2$	$[M+H]^+$ 231.1120	1.5	7	<0.001	0.75	214.0863, 158.0968, 187.1231, 144.0813	Control vs. Beer

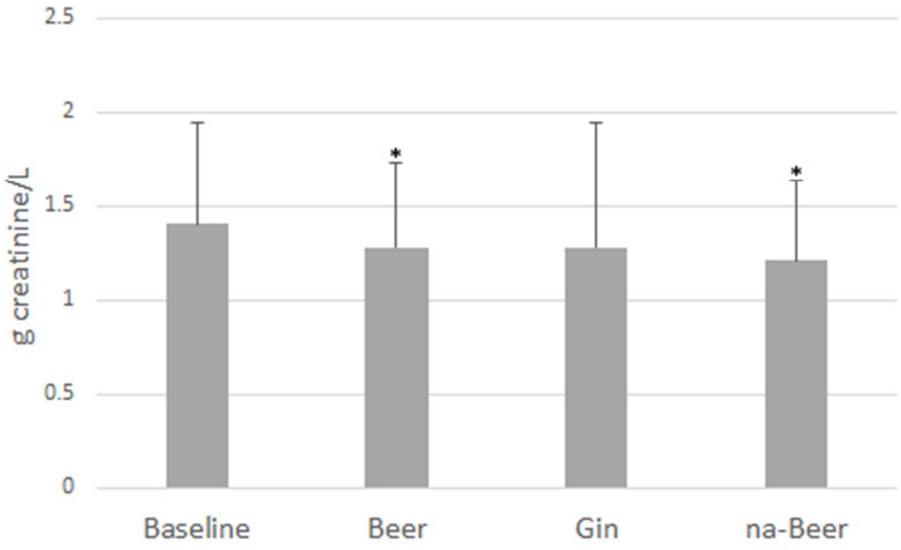
[a]T-test adjusted p-value by false discovery rate


PLS-DA including four classes
t[1]
t[2]
Beer
Control
Gin
na-Beer


PLS-DA Beer vs. Control
t[1]
t[2]
Beer
Control


PLS-DA na-Beer vs. Control
t[1]
t[2]
Control
na-Beer


PLS-DA Gin vs. Control
t[1]
t[2]
Control
Gin



Urinary creatinine excretion at baseline and after 4-weeks of each intervention. /
*Significantly different from before the intervention (Mann Whitney U paired test).

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4.3. Beer, beer polyphenols and cardiovascular diseases

4.3.1. Publication 6. Effects of alcohol and polyphenols from beer on atherosclerotic biomarkers in high cardiovascular risk men: a randomized feeding trial.

Gemma Chiva-Blanch, Emma Magraner, Ximena Condines, Palmira Valderas-Martínez, Irene Roth, Sara Arranz, Rosa Casas, Marta Navarro, Amparo Hervas, Antoni Sisó, Miriam Martínez-Huélamo, Anna Vallverdú-Queralt, **Paola Quifer-Rada**, Rosa Maria Lamuela-Raventos, Ramon Estruch. *Nutrition, Metabolism and Cardiovascular Diseases*, 2015;25(1):36-45.

Summary:

Moderate alcohol consumption exerts a cardioprotective effect, but no studies have evaluated the alcohol-independent cardiovascular effects of the nonalcoholic components of beer. We aimed to evaluate the effects of ethanol and the phenolic compounds of beer on classical and novel cardiovascular risk factors.

Thirty-three high risk male volunteers were included in a randomized, crossover feeding trial. After a washout period, all subjects received beer (30 g alcohol/d, 660 mL), the equivalent amount of polyphenols as non-alcoholic beer (990 mL), and gin (30 g alcohol/d, 100 mL) for 4 weeks. All outcomes were evaluated before and after each intervention period and compliance with the test beers was assessed by measurement of urinary isoxanthohumol. After the run-in period (baseline) and the day after each intervention period, fasting blood, 24-h urine samples and anthropometric measurements were performed with standardized methods, and the blood pressure and heart rate were measured. Laboratory measurements of the samples included plasmatic nitric oxide, blood glucose, total cholesterol and triglycerides, HDL cholesterol, homocysteine, vitamin B_{12}, ApoA-I, ApoA-II, ApoB, lipoprotein (a), insulin, adiponectin, leptin, platelet count, prothrombin time, partial thromboplastin time, and concentrations of factor VII, fibrinogen and plasminogen activator inhibitor-1. Moreover, the expression of several adhesion molecules on the surface of peripheral blood mononuclear cells was analyzed and representative biomarkers of inflammation were quantified.

The results showed that moderate alcohol consumption increased serum HDL-cholesterol, ApoA-I, ApoA-II and adiponectin, and decreased serum fibrinogen, and interleukin 5 concentrations, whereas the non-alcoholic fraction of beer (mainly polyphenols) increased the receptor antagonist of interleukin 1, and decreased lymphocyte expression of lymphocyte function-associated antigen-1, lymphocyte and monocyte expression of Sialil-Lewis X and

monocyte expression of CCR2, and tumor necrosis factor β and interleukin 15 plasma concentrations. No changes were observed in glucose metabolism parameters or in body weight and adiposity parameters.

In conclusion, the phenolic content of beer reduces leukocyte adhesion molecules and inflammatory biomarkers, whereas alcohol mainly improves the lipid profile and reduces some plasma inflammatory biomarkers related to atherosclerosis.

Nutrition, Metabolism & Cardiovascular Diseases (2015) **25**, 36–45

Available online at www.sciencedirect.com

Nutrition, Metabolism & Cardiovascular Diseases

journal homepage: www.elsevier.com/locate/nmcd

Effects of alcohol and polyphenols from beer on atherosclerotic biomarkers in high cardiovascular risk men: A randomized feeding trial

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Received 8 May 2014; received in revised form 18 July 2014; accepted 21 July 2014
Available online 2 August 2014

KEYWORDS
Beer;
Polyphenols;
Alcohol;
Adhesion molecules;
Cytokines;
Cardiovascular disease;
Atherosclerosis

Abstract *Background and aims:* Moderate alcohol consumption exerts a cardioprotective effect, but no studies have evaluated the alcohol-independent cardiovascular effects of the non-alcoholic components of beer. We aimed to evaluate the effects of ethanol and the phenolic compounds of beer on classical and novel cardiovascular risk factors.
Methods and results: Thirty-three high risk male volunteers were included in a randomized, crossover feeding trial. After a washout period, all subjects received beer (30 g alcohol/d, 660 mL), the equivalent amount of polyphenols as non-alcoholic beer (990 mL), and gin (30 g alcohol/d, 100 mL) for 4 weeks. All outcomes were evaluated before and after each intervention period. Moderate alcohol consumption increased serum HDL-cholesterol (~5%), ApoA-I (~6%), ApoA-II (~7%) and adiponectin (~7%), and decreased serum fibrinogen (~8%), and interleukin (IL)-5 (~14%) concentrations, whereas the non-alcoholic fraction of beer (mainly polyphenols) increased the receptor antagonist of IL-1 (~24%), and decreased lymphocyte expression of lymphocyte function-associated antigen-1 (~11%), lymphocyte and monocyte expression of Sialil-Lewis X (~16%) and monocyte expression of CCR2 (~31%), and tumor necrosis factor (TNF)-β (~14%) and IL-15 (~22%) plasma concentrations. No changes were observed in glucose metabolism parameters or in body weight and adiposity parameters.
Conclusion: The phenolic content of beer reduces leukocyte adhesion molecules and inflammatory biomarkers, whereas alcohol mainly improves the lipid profile and reduces some plasma inflammatory biomarkers related to atherosclerosis. Trial registration number: ISRCTN95345245 (http://www.isrctn.org/).

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http://dx.doi.org/10.1016/j.numecd.2014.07.008
0939-4753/

Introduction

Atherosclerosis, the main cause of coronary heart disease (CHD), is considered a low-grade inflammatory disease mediated by the endothelial secretion of chemokines and adhesion molecules, such as integrins and selectins, which recruit circulating monocytes and T-cells to the endothelium and further migrate to the arterial wall triggering atherosclerotic lesions [1].

Moderate alcohol consumption is associated with a decreased cardiovascular risk and mortality independently of the type of alcoholic beverage consumed [2,3]. Nevertheless, red wine, a high polyphenolic fermented beverage, seems to confer greater cardioprotective effects than distilled beverages, which do not contain polyphenols [4], by down-regulating the expression of chemokines and adhesion molecules [5–8]. Recent meta-analyses suggest that beer, a fermented beverage with intermediate polyphenol content, could also confer greater cardioprotection than spirits [9–11], but the results of different trials are controversial, and this question is still under debate [12].

Therefore, we embarked on a randomized, crossover, controlled clinical trial to evaluate and compare the effects of moderate consumption of 30 g alcohol/d of gin, a non-polyphenolic alcoholic beverage, beer, an alcoholic beverage with a medium polyphenolic content, and the same polyphenolic amount of non-alcoholic beer, a medium polyphenolic non-alcoholic beverage, on several biomarkers related to the early stages of atherosclerosis in subjects at high risk for CHD.

Methods

Subjects

A total of 36 male moderate alcohol consumers between 55 and 75 years of age were recruited for the study in the outpatient clinic of the Internal Medicine Department of our institution. Subjects were at high risk for CVD (family history of premature CVD and/or the presence of diabetes, hypertension, dyslipidemia, and overweight/obesity). Exclusion criteria included documented CVD, human immunodeficiency virus infection, chronic liver disease, malnutrition, neoplastic or acute infectious diseases and customary use of vitamin supplements. Participants were offered free beverages but no monetary compensation. The Institutional Review Board of the hospital approved the study protocol, and all participants gave written consent.

Study design and diet monitoring

The study was an open, randomized, controlled, crossover trial with three intervention periods. Two weeks prior to the study, subjects were asked to maintain their usual diet and to refrain from any alcoholic beverage (run-in period). Baseline data were collected after this run-in period. Following this, participants were individually randomized in a crossover design among six sequences of interventions lasting 4 weeks each, in which the test beverages were provided. Randomization was based on a computer-generated random number table, resulting in six possible intervention sequences. Then, participants were instructed to consume beer (660 mL/day, containing 30 g of ethanol and 1209 mg of total polyphenols), non-alcoholic beer (990 mL/day, containing $<$1 g of ethanol and 1243 mg of total polyphenols) or gin (100 mL/day, containing 30 g of ethanol and no polyphenols). No washout periods were included in the study. Therefore, the value of the previous intervention or the baseline value (run-in period) in the first intervention was considered as the starting value of each intervention.

The phenolic profile of the beer, non-alcoholic beer and gin used in the trial was determined by SPE-LC-ESI-MS/MS as previously reported [13,14]. No significant differences were observed in the phenolic content of the daily dose of beer and non-alcoholic beer, while gin contained no detectable phenolic compounds (Supplemental Table 1).

Throughout the study the participants were asked to maintain their usual dietary habits, physical activity level and medications, and to abstain from non-alcoholic beer or alcoholic beverages, except those provided by the investigators. Diet monitoring is explained in the Supplemental Material.

Clinical and laboratory measurements

After the run-in period (baseline) and the day after each intervention period, fasting blood, 24-h urine samples and anthropometric measurements were performed with standardized methods, and the blood pressure (BP) and heart rate were measured 3 times at 5-min intervals on the nondominant arm with an oscillometer (Omron 705 CP; Omron Matsusaka Co Ltd, Matsusaka City, Japan) after 15 min resting in a seated position. The mean of the second and the third measures was considered for statistical analysis.

Serum, EDTA-plasma, and urine samples were stored at -80 °C until assayed.

Compliance with the test beers was assessed by measurement of urinary isoxanthohumol (IX), a biomarker of beer and non-alcoholic beer intake. Briefly, the last day of the run-in period and the last day of each intervention subjects were asked to collect 24-h urine. IX was measured in 24-h urine by SPE-LC-MS/MS as previously described (14).

For the measurement of nitric oxide (NO), the release of NO_2^- and NO_3^-, the stable breakdown products of NO in thawed plasma samples, was determined by a chemiluminescence detector in a NO analyzer (Sievers Instruments, Inc., Boulder, CO).

The following parameters were also determined in thawed samples of whole serum or plasma, as appropriate: blood glucose with the glucose oxidase method; cholesterol and triglycerides with enzymatic procedures; HDL cholesterol after precipitation with phosphotungstic acid and magnesium chloride; and homocysteine and vitamin B12 by an automated electrochemiluminescence

immunoassay system (Advia-Centaur, Siemens, Barcelona, Spain). ApoA-I, ApoA-II, ApoB, lipoprotein (a), insulin, adiponectin and leptin concentrations were quantified in whole serum samples by a customized Human Multi Analyte Profiling assay (Human MAP, Rules Based Medicine Inc., Austin, TX). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated by multiplying fasting insulin concentrations (mIU/L) by fasting glucose concentrations (mM) and dividing by 22.5 [15].

In addition, platelet count, prothrombin time, partial thromboplastin time, and concentrations of factor VII, fibrinogen and plasminogen activator inhibitor-1 (PAI-1) were measured.

Peripheral blood mononuclear cell immunophenotyping

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood by the Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density-gradient. The expression of adhesion molecules on the surface of PBMC was analyzed via double direct immunofluorescence with the use of commercial monoclonal antibodies following the manufacturer's instructions. The adhesion molecules analyzed were as follows: VLA-4 (very late activation antigen-4, CD49-d) (Cytogmos, Barcelona, Spain), LFA-1 (lymphocyte function-associated antigen-1, CD11a) (Bender MedSystems, Vienna, Austria), Mac-1 (CD11b/CD18) (Bender MedSystems), SLe^x (Sialil-Lewis X, CD15s) (Beckman Coulter, Fullerton, CA), CD40 (Caltag Laboratories, Burlingame, CA), CD36 (Beckman Coulter) and CCR2 (R&D Systems, Minneapolis, USA). Fluorescence was monitored with the Sphero™ Rainbow calibration particles (6 peaks) of 6.0–6.4 μm (BD Biosciences, San Jose, CA). Monocytes were identified and selected with the CD14 monoclonal antibody, and T lymphocytes were identified and selected with the CD2 monoclonal antibody (Caltag Laboratories, both). Cell counting (5000 events for T lymphocytes and 3500 for monocytes) and fluorescence analysis were performed in a FACSCalibur Flow Cytometer (Becton–Dickinson, San Jose, CA) using the CellQuest software.

Quantification of soluble biomarkers of inflammation

The following serum soluble adhesion molecules and cytokines and other regulator molecules of adhesion and inflammation processes were quantified by customized Human Multi Analyte Profiling (Human MAP) (Rules Based Medicine Inc., Austin, Texas, USA) following the manufacturer's instructions: CD40 antigen (CD40a), CD40 Ligand (CD40L), C-Reactive Protein (CRP), E-Selectin, Intercellular Adhesion Molecule 1 (ICAM-1), Interleukin-1 receptor antagonist (IL-1ra), IL-3, IL-5, IL-6 receptor (IL-6r), IL-10, IL-13, IL-15, Monocyte Chemotactic Protein 1 (MCP-1), Macrophage-Derived Chemokine (MDC), Monocyte interferon gamma inducing factor (MIG), Regulated on Activation, Normal T cell Expressed and Secreted Protein (RANTES), Tumor Necrosis Factor alpha (TNF-α) Tumor Necrosis Factor beta (TNF- β) and Vascular Cell Adhesion Molecule-1 (VCAM-1).

Statistical analyses

Statistical analyses were performed using the SAS Statistical Analysis System (version 9.2, SAS Institute Inc, Cary, North Carolina). Descriptive statistics [mean ± SD or n(%)] were used to describe the baseline characteristics of the participants and the outcome variables. Variables with a skewed distribution [glucose, HOMA-IR, prothrombin time, CCR2, IL6r, and MCP-1] were transformed to their natural logarithms for analyses and are shown as antilogarithmic values to facilitate the interpretation of the results. To exclude the presence of a carryover effect for the three periods, the interaction between treatment (beer, non-alcoholic beer and gin) and period (1st, 2nd and 3rd) was analyzed by the repeated measures analysis of covariance (ANCOVA) with the baseline values (the values of the previous intervention or the run-in period if the first intervention) as covariates. To analyze the changes within each treatment a Student's t test for paired samples was performed between the data obtained before and after each intervention. One-factor analysis of variance (ANOVA) for repeated measures and the Bonferroni post-hoc test were used to compare the differences of the changes in outcome variables between the interventions. P was considered significant when <0.05.

Results

Characteristics of study subjects and measures of compliance and dietary control

Of the 36 subjects included, three withdrew before completing the study. The reasons for withdrawal were work-related ($n = 2$) and need to travel ($n = 1$). Therefore, 33 subjects completed the study. The baseline characteristics are shown in Table 1. There were no individual deviations from the interventions according to the participants' dietary reports. Protocol adherence was optimum in all subjects according to their self reports. As a measure of intervention compliance, IX -a biomarker of beer consumption [13,14]- was determined in 24-h urine samples collected the last day of the run-in period and the last day of each intervention. After the consumption of beer and non-alcoholic beer, 24-h urinary excretion of IX increased to 7.2 ± 3.3 and 7.5 ± 2.9 μg, respectively (with no significant differences between the two values), whereas it was not detected at baseline and after the gin intervention ($P < 0.001$ between the two beer interventions and the gin and baseline interventions). According to these findings, compliance was excellent.

No significant differences were observed in energy and nutrient intake (Supplemental Table 2) or energy expenditure in physical activity before and after each intervention according to food records and physical activity questionnaires. No individual changes in drug intake were reported and no adverse effects were observed. No carryover effect was observed for any outcome. In addition, we compared the differences between the values obtained after each intervention period

Table 1 Baseline characteristics of the 33 subjects included in the study.

	Mean ± SD[a]
Age (years)	61 ± 6
Hypertension [*n*(%)]	21 (64)
Dyslipemia [*n*(%)]	23 (70)
Type 2 Diabetes Mellitus [*n*(%)]	7 (21)
Current smokers [*n*(%)]	8 (24)
Sedentarism [*n*(%)]	6 (18)
Family history of premature CHD [*n*(%)]	2 (6)
Medications [*n*(%)]	
ACE Inhibitors	16 (48)
Diuretics	4 (12)
Statins	15 (45)
Fibrates	1 (3)
Oral hypoglycemic drugs	6 (18)
Aspirin or antiplatelet drugs	4 (12)
Body weight (kg)	85.6 ± 12.8
BMI (kg/m2)	28.8 ± 4.1
BMI≥ 25 kg/m2 [*n*(%)]	28 (85)
Abdominal circumference (cm)	101 ± 10
WHR	0.95 ± 0.05
Systolic Blood Pressure (mmHg)	138 ± 16
Diastolic Blood Pressure (mmHg)	81 ± 8
Heart rate (beats/min)	68 ± 11
Glucose (mg/dL)	112 ± 27
Triglycerides (mg/dL)	99 ± 43
Total cholesterol (mg/dL)	185 ± 31
LDL cholesterol (mg/dL)	119 ± 26
HDL cholesterol (mg/dL)	44 ± 11
LDLc/HDLc ratio	3.03 ± 0.88
Folic acid (serum) (ng/mL)	8.7 ± 3.7
Intraerythrocytary folic acid (ng/mL)	407 ± 94
Vitamin B12 (pg/mL)	432 ± 206
Albumin (mg/mL)	43 ± 2
ASAT (UI/L)	25 ± 12
ALAT (UI/L)	29 ± 16
GGT (UI/L)	29 ± 14

CHD, Coronary Heart Disease; BMI, Body Mass Index; WHR, waist-to-hip ratio; ACE, Angiotensin-Converting Enzyme; LDL, Low Density Lipoprotein; HDL, High Density Lipoprotein; ASAT, Aspartate aminotransferase; ALAT, Alanine aminotransferase; GGT, Gamma glutamyl transpeptidase.

[a] Mean ± SD or *n* (%), when indicated ($n = 33$).

and the baseline value (after the run-in period) by repeated measures ANOVA and the results did not significantly change.

Effects on blood pressure and plasma nitric oxide concentration

As shown in Fig. 1, systolic BP decreased a mean of 4 mm Hg after the non-alcoholic beer intervention ($P = 0.007$), while no differences were observed after the beer and gin interventions. Diastolic BP, heart rate and plasma NO remained constant throughout the study.

Effects on anthropometric parameters, glucose metabolism, lipid profile and adipokines

Changes in anthropometric parameters, glucose metabolism, adipokines, lipid profile and other biochemical parameters are shown in Table 2. After the beer and gin interventions, HDL cholesterol, ApoA-I, ApoA-II and adiponectin increased by ~5, ~6%, ~7% and ~7%, respectively, and also compared to the non-alcoholic beer intervention. Apo A-I and Apo A-II also decreased by ~0.5 and ~6%, respectively, after the non-alcoholic beer intervention. Homocysteine concentration significantly decreased by ~6% and serum folic acid increased by ~9% only after the non-alcoholic beer intervention. No significant changes were observed before and after each intervention or among the three interventions for body weight, BMI, waist-to-hip ratio, and glucose metabolism parameters.

Effects on coagulation factors

Serum fibrinogen decreased by ~8% after the beer and gin interventions, compared to the non-alcoholic beer intervention ($P = 0.005$, Supplemental Table 3). No differences were observed in platelet count, prothrombin and partial thromboplastin times, factor VII and PAI-1 concentrations in any of the interventions.

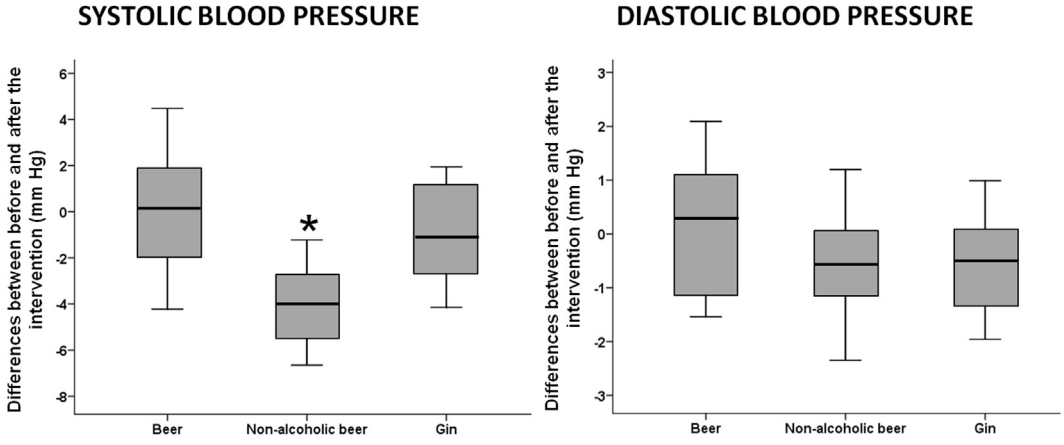

Figure 1 Changes in systolic and diastolic blood pressure in the 33 subjects studied after the three interventions. *$P = 0.007$ (*t* test between before and after the intervention).

Table 2 Changes in glucose control, lipid profile and other cardiovascular risk factors after the three interventions in the 33 study subjects.

	Beer intervention			Non-alcoholic beer intervention			Gin intervention			P^c
	Mean ± SD[a]		Mean differences (95% CI)[b]	Mean ± SD[a]		Mean differences (95% CI)[b]	Mean ± SD[a]		Mean differences (95% CI)[b]	
	Before	After		Before	After		Before	After		
Antropometric parameters										
Body weight (Kg)	85.4 ± 12.8	86.0 ± 12.1	0.62 (−0.19, 1.44)	86.0 ± 13.1	85.8 ± 13.0	−0.17 (−0.88, 0.53)	86.0 ± 13.0	85.9 ± 12.9	−0.1 (−0.79, 0.58)	0.390
BMI(kg/m^2)	28.8 ± 4.0	29.0 ± 3.8	2.06 (−0.01, 4.14)	28.9 ± 4.1	28.9 ± 4.0	0.81 (−2.58, 4.2)	29.0 ± 4.0	29.0 ± 4.0	−1.16 (−4.10, 1.78)	0.280
Waist-to-hip ratio	0.96 ± 0.05	0.96 ± 0.05	0 (−0.007, 0.006)	0.96 ± 0.05	0.95 ± 0.06	0 (−0.008, 0.001)	0.95 ± 0.05	0.96 ± 0.05	0 (−0.005, 0.009)	0.497
Glucose metabolism										
Glucose (mg/dL)	110 ± 25	112 ± 27	2.13 (−4.16, 8.41)	112 ± 28	109 ± 29	−3.47 (−9.33, 2.4)	114 ± 32	112 ± 27	−2.84 (−8.69, 3)	0.444
Insulin (μIU/mL)	3.31 ± 1.96	3.76 ± 2.49	0.44 (−0.26, 1.15)	3.6 ± 4	3.51 ± 1.99	−0.07 (−1.27, 1.13)	4.2 ± 3.63	3.98 ± 4.31	−0.22 (−1.05, 0.62)	0.634
HOMA-Insulin resistance	0.93 ± 0.68	1.13 ± 1.03	0.21 (−0.09, 0.5)	1.16 ± 2.01	0.98 ± 0.71	−0.18 (−0.76, 0.41)	1.33 ± 1.61	1.26 ± 2.09	−0.07 (−0.45, 0.32)	0.524
Lipids, lipoproteins and apolipoproteins										
Total cholesterol (mg/dL)	191 ± 32	189 ± 28	−2.19 (−8.62, 4.24)	189 ± 32	191 ± 31	2.66 (−3.15, 8.47)	187 ± 29	191 ± 30	3.66 (−2.49, 9.80)	0.433
Triglycerides (mg/dL)	100 ± 43	110 ± 50	10.41 (−1.83, 22.65)	107 ± 54	102 ± 42	−4.97 (−18.48, 8.55)	103 ± 41	107 ± 50	4.25 (−5.88, 14.38)	0.289
LDL-cholesterol (mg/dL)	123 ± 28	119 ± 27	−3.97 (−0.46, 8.39)	120 ± 25	124 ± 29	3.81 (−1.65, 9.28)	120 ± 27	123 ± 27	3.63 (−3.08, 10.33)	0.227
HDL-cholesterol (mg/dL)	44.1 ± 11.2	45.5 ± 10.8	1.44 (0.24, 3.11)[d,1]	43.7 ± 10.8	42.8 ± 10.4	−1.13 (−2.22, 0.03)[2]	43.2 ± 10.3	45.5 ± 11.2	2.22 (0.64, 3.79)[d,2]	0.009
Lipoprotein(a) (mg/dL)	547 ± 504	529 ± 440	−18.25 (−62.05, 25.56)	496 ± 442	515 ± 440	19.33 (−19.04, 57.71)	561 ± 537	514 ± 489	−46.17 (−95.39, 3.05)	0.133
Apolipoprotein A-I (mg/dL)	1.57 ± 0.5	1.79 ± 0.24	0.22 (0.02, 0.42)[d,1]	1.76 ± 0.27	1.67 ± 0.23	−0.09 (−0.16, −0.01)[d,2]	1.68 ± 0.25	1.77 ± 0.25	0.1 (0.02, 0.17)[d,1]	0.029
Apolipoprotein A-II (ng/mL)	256 ± 40	270 ± 41	14.68 (1.13, 28.23)[d,1]	274 ± 43	257 ± 44	−17.43 (−29.06, −5.80)[d,2]	255 ± 43	274 ± 52	18.71 (5.22, 32.34)[d,1]	0.004
Apolipoprotein B (mg/dL)	100 ± 20	99 ± 20	−1.06 (−5.43, 3.31)	99 ± 22	100 ± 19	0.91 (−4.13, 5.94)	98 ± 18	100 ± 19	1.53 (−2.65, 5.71)	0.763
Other cardiovascular risk factors										
Homocysteine (μmol/L)	10.0 ± 3.2	10.6 ± 2.6	0.3 (−0.11, 0.72)	10.9 ± 2.3	10.4 ± 2.1	−0.66 (−1.28, −0.05)[d]	10.7 ± 2.4	10.7 ± 2.5	0.11 (−0.66, 0.88)	0.110
Vitamin B_{12} (pg/mL)	404 ± 189	392 ± 243	−6.28 (−41.36, 28.81)	384 ± 221	381 ± 179	−8.17 (−49.86, 33.52)	409 ± 222	390 ± 213	−24.41 (−62.44, 13.61)	0.821
Folic acid, serum (ng/mL)	8.28 ± 3.73	8.22 ± 3.12	−0.02 (−0.97, 0.94)	8.45 ± 3.62	9.23 ± 4.21	0.77 (0.11, 1.56)[d]	8.00 ± 3.45	7.97 ± 3.5	−0.04 (−0.86, 0.78)	0.235
Adipokines										
Leptin (ng/mL)	9.61 ± 5.49	10.37 ± 5.97	0.86 (−0.11, 1.82)	9.04 ± 5.03	9.29 ± 5.17	0.25 (−0.72, 1.22)	10.24 ± 6.34	10.35 ± 6.29	0.11 (−0.73, 0.95)	0.470
Adiponectin (μg/mL)	3.26 ± 1.34	3.47 ± 1.47	0.21 (0.04, 0.38)[d,1]	3.28 ± 1.32	3.20 ± 1.33	−0.08 (−0.25, 0.11)[2]	3.08 ± 1.21	3.30 ± 1.31	0.22 (0.06, 0.38)[d,1]	0.017

BMI, Body Mass Index; HOMA-Insulin resistance, Homeostasis Model Assessment of Insulin resistance; LDL, Low Density Lipoprotein; HDL, High Density Lipoprotein.

[a] The result expressed as mean ± SD ($n = 33$).

[b] The result expressed as mean differences (95% CI) between after and before each intervention. Before each intervention is the value of the previous intervention or the baseline value (run-in period) in the first intervention.

[c] The result expressed as P value of the repeated-measures ANOVA from the differences between interventions.

[d] Significant differences ($P < 0.05$) between after and before the intervention, measured by the Student's t test for paired samples. Values in a row with different superscript numbers are significantly different ($P < 0.05$, Bonferroni post-hoc test).

Table 3 Changes in the expression of adhesion molecules on the surface of T lymphocytes and monocytes after the three interventions in the 33 study subjects.

	Beer intervention			Non-alcoholic beer intervention			Gin intervention			P^c
	Mean ± SD[a]		Mean differences (95% CI)[b]	Mean ± SD[a]		Mean differences (95% CI)[b]	Mean ± SD[a]		Mean differences (95% CI)[b]	
	Before	After		Before	After		Before	After		
T lymphocytes										
LFA-1 (MFI[d])	135.69 ± 41.34	120.56 ± 38.29	−17.29 (−30.11, −4.47)[e,1]	140.72 ± 43.96	127.39 ± 43.98	−16.17 (−25.43, −6.91)[e,1]	136.42 ± 47.07	134.11 ± 45.50	−1.42 (−8.40, 5.56)[2]	0.031
Mac-1 (MFI)	76.14 ± 34.36	74.17 ± 45.28	−3.81 (−22.4, 14.78)	81.88 ± 35.21	74.63 ± 39.44	−11.54 (−26.74, 3.66)	83.66 ± 43.82	79.64 ± 47.93	−0.13 (−13.7, 13.44)	0.637
VLA-4 (MFI)	44.78 ± 13.56	44.62 ± 13.79	0.03 (−3.96, 4.01)	45.4 ± 11.6	44.36 ± 12.62	−1.28 (−3.94, 1.37)	45.39 ± 12.51	44.68 ± 13.65	0.13 (−2.27, 2.52)	0.807
SLe^x (MFI)	135.58 ± 82.62	109.21 ± 89.84	−28.68 (−54.972, −2.4)[e]	150.56 ± 104.13	123.72 ± 95.07	−23.59 (−52.367, −5.19)[e]	139.51 ± 85.81	121.37 ± 102.5	−17.59 (−39.623, 4.44)	0.865
CD40 (MFI)	95.57 ± 73.67	86.78 ± 86.62	−6.92 (−32.77, 18.93)	96.91 ± 80.15	90.71 ± 83.17	−2.82 (−28.33, 22.7)	108.51 ± 91.2	91.92 ± 84.38	−6.5 (−29.84, 16.85)	0.975
Monocytes										
LFA-1 (MFI)	70.89 ± 34.65	73.49 ± 21.25	0.7 (−8.52, 9.91)	77.14 ± 28.2	74.86 ± 23.17	−5.81 (−17.03, 5.41)	80.95 ± 24.4	75.97 ± 25.51	−4.98 (−12.47, 2.51)	0.638
Mac-1 (MFI)	40.46 ± 11.1	40.3 ± 13.99	−1.04 (−4.58, 2.49)	40.14 ± 10.09	40.27 ± 10.13	1.7 (−1.99, 5.39)	40.46 ± 10.17	40.06 ± 11.27	0.3 (−3.37, 3.97)	0.646
VLA-4 (MFI)	31.91 ± 9.82	32.4 ± 9.76	−0.19 (−2.79, 2.41)	31.29 ± 8.31	31.13 ± 10.22	0.2 (−2.15, 2.54)	33.43 ± 9.03	31.35 ± 10.03	−2.12 (−4.89, 0.65)	0.493
SLe^x (MFI)	55.05 ± 23.16	51.19 ± 21.6	−4.15 (−9.495, −0.58)[e]	54.58 ± 21.92	50.45 ± 22.96	−3.53 (−8.063, −0.81)[e]	55.63 ± 19.67	51.61 ± 22.98	−3.79 (−9.085, 1.5)	0.989
CD40 (MFI)	33.45 ± 14.55	30.57 ± 15.14	−3.56 (−8.39, 1.27)	32.86 ± 16.3	30.09 ± 14.93	−2.90 (−9.38, 3.59)	33.14 ± 14.14	31.67 ± 16.01	0.49 (−3.58, 4.56)	0.583
CD36 (MFI)	860.05 ± 614.6	806.2 ± 572.6	−54.73 (−238.35, 128.88)	915.57 ± 641.43	784.79 ± 604.17	−130.77 (−272.10, 10.55)	897.34 ± 642.9	787.60 ± 639.61	−109.74 (−260.99, 41.51)	0.733
CCR2 (MFI)	94.96 ± 82.70	68.36 ± 44.49	−26.59 (-54.62, −1.43)[e,1]	110.40 ± 82.54	70.61 ± 52.06	−39.79 (−60.30, −19.28)[e,1]	80.13 ± 58.04	97.71 ± 67.60	11.57 (−6.42, 29.56)[2]	0.011

LFA-1, Lymphocyte Function-Associated Antigen-1; Mac-1, Macrophage-1 antigen; VLA-4, Very Late Antigen-4; SLe^x, Sialil-Lewis X.

[a] The results expressed as mean ± SD ($n = 33$).

[b] The results expressed as mean differences (95% CI) between after and before each intervention. Before each intervention is the value of the previous intervention or the baseline value (run-in period) in the first intervention.

[c] The results expressed as P value of the repeated-measures ANOVA from the differences between interventions.

[d] The results expressed as Mean Fluorescence Intensity (arbitrary units).

[e] Significant differences ($P < 0.05$) between after and before the intervention, measured by the Student's t test for paired samples. Values in a row with different superscript numbers are significantly different ($P < 0.05$, Bonferroni post-hoc test).

Table 4 Changes in circulating inflammatory biomarkers after the three interventions in the 33 study subjects.

	Beer intervention			Non-alcoholic beer intervention			Gin intervention			P^c
	Mean ± SD[a]		Mean differences (95% CI)[b]	Mean ± SD[a]		Mean differences (95% CI)[b]	Mean ± SD[a]		Mean differences (95% CI)[b]	
	Before	After		Before	After		Before	After		
CD40a (ng/mL)	0.77 ± 0.14	0.81 ± 0.16	0.04 (−0.01, 0.1)	0.82 ± 0.19	0.82 ± 0.2	0.01 (−0.05, 0.07)	0.78 ± 0.18	0.81 ± 0.16	0.03 (−0.02, 0.08)	0.738
CD40L (ng/mL)	1.43 ± 1	1.69 ± 1.24	0.26 (−0.16, 0.68)	1.83 ± 1.4	1.89 ± 1.27	0.11 (−0.20, 0.42)	1.43 ± 1.08	1.7 ± 1.05	0.27 (−0.05, 0.6)	0.780
CRP (μg/mL)	2.93 ± 2.48	2.52 ± 2.62	−0.65 (−1.76, 0.46)	2.58 ± 2.52	2.53 ± 1.63	−0.01 (−0.77, 0.75)	3.34 ± 3.22	2.85 ± 2.99	0.17 (−0.61, 0.94)	0.496
E-Selectin (ng/mL)	7.59 ± 3.82	7.84 ± 3.72	0.24 (−0.05, 0.54)[1,2]	7.64 ± 3.59	7.3 ± 3.67	−0.33 (−0.71, 0.04)[2]	7.00 ± 3.24	7.36 ± 3.39	0.36 (0.01, 0.71)[d,1]	0.005
ICAM-1 (ng/mL)	127 ± 29	134 ± 36	7.11 (−2.37, 16.59)	134 ± 41	131 ± 29	−0.55 (−10.43, 9.32)	132 ± 33	131 ± 37	−1.68 (−8.23, 4.88)	0.399
IL-1ra (pg/mL)	75.1 ± 35.3	93.1 ± 38.4	17.97 (1.53, 34.4)[d,1]	90.7 ± 54.3	95.1 ± 47.9	5.89 (−18.31, 30.09)[1,2]	90.9 ± 46.1	81.4 ± 33.4	−10.61 (−26.31, 5.09)[2]	0.050
IL-3 (ng/mL)	0.09 ± 0.05	0.08 ± 0.04	−0.01 (−0.02, 0.01)	0.09 ± 0.05	0.09 ± 0.05	0.01 (−0.01, 0.02)	0.09 ± 0.04	0.08 ± 0.04	−0.01 (−0.02, 0)	0.338
IL-5 (pg/mL)	27.9 ± 16.5	23.8 ± 11.3	−4.02 (−7.56, −0.46)[d,1]	25.2 ± 15.6	27.2 ± 14.8	2.06 (−1.22, 5.34)[2]	24.6 ± 10.6	21.6 ± 12.6	−2.94 (0.73, 6.61)[d,1]	0.043
IL-6r (ng/mL)	30.3 ± 7.9	30.4 ± 8.7	0.12 (−1.15, 1.38)	30.5 ± 8.6	29.6 ± 7.9	−0.9 (−1.86, −0.06)[d]	29.9 ± 8.4	30.1 ± 8.3	0.18 (−0.80, 1.15)	0.387
IL-10 (pg/mL)	4.11 ± 1.42	4.32 ± 1.36	0.21 (−0.51, 0.93)	4.59 ± 1.25	4.2 ± 1.68	−0.38 (−1.12, 0.36)	4.21 ± 1.63	4.59 ± 1.97	0.38 (−0.30, 1.06)	0.521
IL-13 (pg/mL)	38.4 ± 17.0	36.6 ± 12.9	−1.89 (−5.85, 2.08)	37.0 ± 16.5	38.6 ± 16.5	1.56 (−1.75, 4.88)	37.1 ± 12.5	36.8 ± 16.1	−0.3 (−3.69, 3.09)	0.477
IL-15 (ng/mL)	0.24 ± 0.14	0.22 ± 0.11	−0.03 (−0.08, 0.03)[1,2]	0.23 ± 0.1	0.19 ± 0.08	−0.05 (−0.09, −0.01)[d,1]	0.21 ± 0.13	0.29 ± 0.14	0.07 (0.01, 0.13)[d,2]	0.043
MCP-1 (pg/mL)	487 ± 345	495 ± 360	7.97 (−27.42, 43.35)	493 ± 353	504 ± 345	12.34 (−21.95, 46.64)	508 ± 378	506 ± 377	−2.09 (−48.33, 44.15)	0.894
MDC (pg/mL)	507 ± 111	497 ± 106	−9.41 (−43.73, 24.92)	505 ± 116	511 ± 133	7.66 (−25.08, 40.39)	519 ± 112	511 ± 91	−7.88 (−38.56, 22.81)	0.774
MIG (pg/mL)	1265 ± 637	1445 ± 1190	183.5 (−173.0, 540.0)	1251 ± 536	1670 ± 839	196.6 (−60.6, 453.7)	1250 ± 629	1280 ± 534	30.5 (−146.0, 207.0)	0.518
RANTES (ng/mL)	23.8 ± 6.7	23.4 ± 7.6	−0.38 (−2.01, 1.24)	24.1 ± 7.1	21.1 ± 7.5	−3.20 (−5.66, −0.74)[d]	23.2 ± 6.0	22.7 ± 7.3	−0.51 (−3.13, 2.12)	0.212
TNF-α (pg/mL)	22.0 ± 11.1	23.6 ± 13.5	1.56 (−1.44, 4.57)	23.9 ± 15.4	24.0 ± 13.8	0.21 (−3.04, 3.47)	22.6 ± 12.9	22.4 ± 12.0	−0.2 (−3.49, 3.09)	0.764
TNF-β (pg/mL)	13.9 ± 7.5	11.1 ± 5.7	−3.2 (−7.31, 0.92)[1]	11.0 ± 8.7	11.1 ± 5.7	−1.61 (−5.11, 1.89)[1]	10.1 ± 6.4	13.7 ± 6.8	3.63 (0.61, 6.65)[d,2]	0.032
VCAM-1 (ng/mL)	461 ± 93	464 ± 96	2.38 (−10.63, 15.38)	451 ± 95	452 ± 91	0.93 (−13.04, 14.91)	466 ± 98	456 ± 107	−10.5 (−28.79, 7.79)	0.349

CD40a, CD40 antigen; CD40L, CD40 Ligand; CRP, C-Reactive Protein; ICAM-1, Intercellular Adhesion Molecule 1; IL, Interleukin; IL-1ra, Interleukin −1 receptor antagonist; IL-6r, Interleukin −6 receptor; MCP, Monocyte Chemotactic Protein; MDC, Macrophage-Derived Chemokine; MIG, Monokine Induced by Gamma Interferon; RANTES, Regulated on Activation, Normal T Cell Expressed and Secreted; TNF, Tumor Necrosis Factor alpha; VCAM-1, Vascular Cell Adhesion Molecule-1.

[a] The results expressed as mean ± SD ($n = 33$).

[b] The results expressed as mean differences (95% CI) between after and before each intervention. Before each intervention is the value of the previous intervention or the baseline value (run-in period) in the first intervention.

[c] The results expressed as P value of the repeated-measures ANOVA from the differences between interventions.

[d] Significant differences ($P < 0.05$) between after and before the intervention, measured by the Student's t test for paired samples. Values in a row with different superscript numbers are significantly different ($P < 0.05$, Bonferroni post-hoc test).

Expression of serum and cell adhesion molecules

As observed in Table 3, after the alcoholic and non-alcoholic beer interventions lymphocyte expression of LFA-1 and SLe^x decreased (~11 and 16%, respectively), as did monocyte expression of SLe^x and CCR2 (~16 and 31%, respectively). Table 4 shows that serum concentrations of IL-1ra increased (~24%) and IL-5 decreased (~14%) after beer and gin interventions, whereas E-Selectin, IL-6r, IL-15, RANTES and TNF-β only decreased after the non-alcoholic beer intervention (~4, 3, 22, 13 and 14% respectively).

Discussion

Moderate beer intake may exert higher protection against CHD than spirits [9–11]. However, even in prospective cohort studies it is difficult to assess the type and amount of alcohol consumed by the subjects and to control important confounding factors such as diet and exercise. In fact, the issue of nutrition and physical activity can only be solved in well-designed randomized clinical trials. In the current study, we have carefully monitored food intake with a 7-d food recall questionnaire, exercise with the Minnesota Leisure Time Physical Activity Questionnaire and interventions by measurement of IX in urine. According to these results, the changes observed between interventions could be attributed to beer (constituted mainly by alcohol plus polyphenols), non-alcoholic beer (polyphenols) and gin (alcohol).

Low plasma levels of HDL cholesterol are a strong, independent risk factor for cardiovascular disease. As expected [3,16], in the current trial ethanol consumption increased plasma HDL cholesterol, ApoA-I, and ApoA-II concentrations and decreased serum fibrinogen concentrations since these changes were observed only after the beer and gin interventions, but not after the non-alcoholic beer intervention. Therefore, beer polyphenols do not affect HDL cholesterol secretion, in the line with Nicod et al., who observed that red wine, cocoa and green tea polyphenols neither increased cholesterol secretion by intestinal cells nor enhanced HDL functionality [17].

On the other hand, systolic BP, homocysteine and several biomarkers of inflammation decreased only after the non-alcoholic beer intervention, and these effects should be attributed to the non-alcoholic fraction of the beer, mainly polyphenols. Thus, no synergistic effects were observed between the alcoholic and the non-alcoholic fraction of beer in any of the outcomes studied, but to the contrary, in our study, alcoholic beer did not exhibit some beneficial effects observed after non-alcoholic beer, suggesting a possible antagonistic effect between alcohol and the non-alcoholic fraction of beer.

The effects of moderate alcohol consumption on BP are controversial and may be dependent on gender and grade of endothelial dysfunction. As we observed in a previous study [18], after one month of intervention moderate alcohol consumption did not affect BP in high cardiovascular risk subjects. By contrast, the systolic BP decreased 4 mmHg after non-alcoholic beer consumption in the study population, being of major clinical significance since this decrease has been associated with a 12% and 16% reduction in CHD and stroke risks, respectively [19]. This effect may be due to the vasodilator properties of polyphenols [20]. However, this reduction is approximately two-thirds the lowering effect observed after dealcoholized red wine intake [21]. In our study moderate alcohol intake as beer or gin did not modify HOMA-IR, as reported previously [21–23]. Neither did the non-alcoholic beer intervention have any effect on the HOMA-IR, similar to the results of Beulens et al. [21], who, after comparison of the effects of beer and red wine, observed that beer does not improve HOMA-IR in high cardiovascular risk patients, while red wine intake showed a protective effect on insulin resistance [24–26]. Interestingly, adiponectin concentrations diminished after beer intake, similar to the findings obtained in the same study of Beulens et al. [21].

To our knowledge this is the first time that the effects of moderate beer consumption and its fractions on the expression of leukocyte adhesion molecules have been evaluated. We observed that the non-alcoholic fraction of beer was responsible for decreasing the leukocyte expression of adhesion molecules. Previous studies, based on the basis of the same daily amount of alcohol, have reported the inhibitory effects of wine intake on the expression of serum inflammatory and leukocyte adhesion molecules by increasing the serum concentration of IL-10 and decreasing ICAM-1,IL-1α, IL-6, E- and P-Selectin, CD40, MCP-1, MDC and VCAM-1, and the lymphocyte expression of VLA-4, LFA-1and SLex, and monocyte expression of Mac-1, SLex, MCP-1 and CCR2 [5,7,8,27]. However, although the cardioprotective effects of moderate beer intake observed in the current study (increased serum concentrations of IL-1ra, decreased IL-5, and decreased lymphocyte expression of LFA-1 and SLe^x and monocyte expression of SLe^x and CCR2) are higher than those of gin, when we compared these results with those observed after moderate wine intake [4,5], the protective effects of moderate wine intake (especially red wine) were higher than those observed after beer intake, suggesting that the non-alcoholic fraction of beer may be less cardioprotective than that of wine [9–11].

Xanthohumol and related prenylated flavonoids, polyphenols almost exclusively present in hop-derived products such as beer, have shown an anti-inflammatory effect *in vitro* [28]. *In vivo*, we observed an anti-inflammatory effect after beer and non-alcoholic beer interventions through the decrease in serum concentrations of inflammatory biomarkers, effects that should be related to the non-alcoholic components of beer, mainly polyphenols.

This study has some limitations. No washout periods were made between the interventions, because this would have prolonged the study 6 weeks more, making it difficult to ensure compliance, and the subjects would have been more inclined to withdraw from the study. However, since previous studies have shown that changes in cellular and soluble adhesion molecules are already observed after 2 weeks of intervention [8,27] and have also shown a lack of

carryover effect [5], the presence of a washout period would probably not have changed the results obtained. Another limitation is that the dealcoholization process of beer provokes a significant loss of non-alcoholic compounds such as polyphenols and other bioactive compounds as a result of the different composition of the two beers. We tried to amend this inconvenience by equalizing the amount of polyphenols in both interventions (alcoholic and non-alcoholic beer), increasing 330 mL the non-alcoholic beer dose with respect to the alcoholic beer. Additionally, gin may contain some substances derived from the aging process as juniper components. Given the low bioavailability of these compounds, and that gin contains no detectable polyphenols, it is possible to assume that gin contains no interfering substances other than ethanol. Furthermore, the consumption of alcohol was not blinded, which is difficult to achieve given the distinct taste of alcoholic beverages. In addition, our study sample was composed of older men at high cardiovascular risk, thus, the results may not be extrapolated to other populations. Lastly, the duration of this study was of 4 weeks which may not represent the potential beneficial effects of long-term moderate alcohol consumption.

In conclusion, moderate beer and non-alcoholic beer intake confers greater protective effects on the cardiovascular system than distilled beverages probably because of their polyphenolic content.

Acknowledgments

We are grateful for the collaboration of the participants. We are indebted to the Asociación de Cerveceros de España for providing the non-alcoholic and regular beers, and Gin Xoriguer for providing the gin used in this study. CIBEROBN is an initiative from the Instituto de Salud Carlos III. This work was developed at the Centre de Recerca Biomèdica Cellex, Barcelona, Spain. Supported by grants from The European Foundation for Alcohol Research (ERAB) EA 11 17, CICYT (AGL2010-22319-C03), the Spanish Ministry of Science and Innovation (MICINN), and CIBEROBN, an initiative of the Instituto de Salud Carlos III. G C-B and P Q-R thank the Manuel de Oya fellowship program. P.V-M thanks APIF fellowship program from the University of Barcelona, S. A thanks the Sara Borrell postdoctoral program (CD10/00151) supported by the Instituto de Salud Carlos III and M M-H thanks the predoctoral program of MICINN.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.numecd.2014.07.008.

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4.3.2. Publication 7. Moderate alcohol intake is inversely associated with total mortality and cardiovascular events in an old population. Specific effects depending on the beverage consumed and the diet followed.

Paola Quifer-Rada, Anna Tresserra-Rimbau, Alexander Medina-Remón, Dolores Corella, Emilio Ros,Miguel A. Martínez-González, Montse Fitó, Jordi Salas-Salvadó, Rafael de la Torre, José Lapetra, Fernando Arós, Miquel Fiol, Lluis Serra-Majem, Xavier Pintó, Miguel Angel Muñoz, Enrique Gómez-Gracia, Alfredo Gea , José V. Sorlí, Nancy Babio, Emanuel Rubin, Rosa M. Lamuela-Raventós, and Ramón Estruch. To be submitted in JAMA.

Summary:

Moderate alcohol consumption is inversely associated with incident cardiovascular disease in observational studies. It has been suggested that fermented beverages such as red wine and beer may confer greater protective effects than spirits owing to their higher polyphenolic content. In this work, we aimed to evaluate associations of moderate intake of wine, beer and spirits with all-cause and specific mortality and incidence of cardiovascular events.

The randomized controlled PREvencion con DIeta MEDiterránea (PREDIMED) trial testing Mediterranean diet against a low-fat control diet for cardiovascular diseases prevention was analyzed as an observational cohort. From 2003 to 2010, 7447 old participants (mean age 67±6 years) at high cardiovascular risk were enrolled and followed for a mean of 4.8 years. Consumption of alcoholic beverages was assessed at baseline and yearly during follow-up with a validated food frequency questionnaire. Total mortality and the occurrence of major cardiovascular events (myocardial infarction, stroke, and death from cardiovascular causes) were monitored.

Complete data were available for 7154 participants. We found an inverse association between total alcohol intake and all-cause mortality [Hazard Ratio (HR): 0.73 (95% Confidence Interval, 0.59-0.91)]. Compared to non-drinkers, light (< 5 g alcohol/day) and moderate (5-15 g alcohol/day) drinking of total wine, red wine, and beer was inversely associated with all-cause mortality, with HRs of 0.70 (0.56-0.88), 0.62 (0.49-0.78), and 0.62 (0.67-0.84), respectively. Wine was associated with a lower incidence of cardiovascular events at moderate and high (>15-70 g alcohol/day) consumption levels, with HRs of 0.73 (0.51-0.99) and 0.29 (0.18-0.47), respectively. Light and moderate beer consumption was also associated with a lower incidence of cardiovascular events [HR: 0.75 (0.62-0.88)]. Alcohol intake was associated with reduced

cardiovascular and cancer mortality in participants assigned the Mediterranean diet, but not in those allocated the control, low-fat diet.

In conclusion, in an older Mediterranean population at high cardiovascular risk, light and moderate consumption of alcoholic beverages, mainly red wine and beer, was inversely associated with total mortality and major cardiovascular events.

Moderate alcohol intake is inversely associated with total mortality and cardiovascular events in an old population. Specific effects depending on the beverage consumed and the diet followed

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ABSTRACT

Importance: Moderate alcohol consumption is inversely associated with incident cardiovascular disease in observational studies. It has been suggested that fermented beverages such as red wine and beer may confer greater protective effects than spirits owing to their higher polyphenolic content.

Objective: To evaluate associations of moderate intake of wine, beer and spirits with all-cause and specific mortality and incidence of cardiovascular events.

Design: The randomized controlled PREvencion con DIeta MEDiterránea (PREDIMED) trial testing Mediterranean diet against a low-fat control diet for cardiovascular diseases prevention was analyzed as an observational cohort. From 2003 to 2010, 7447 participants at high cardiovascular risk were enrolled and followed for a mean of 4.8 years.

Setting: Primary care centers affiliated to 10 teaching Hospitals.

Participants: Older individuals (mean age 67±6 y) at high cardiovascular risk.

Exposure: Consumption of alcoholic beverages was assessed at baseline and yearly during follow-up with a validated food frequency questionnaire.

Main Outcomes and Measures: Total mortality and the occurrence of major cardiovascular events (myocardial infarction, stroke, and death from cardiovascular causes).

Results: Complete data were available for 7154 participants. We found an inverse association between total alcohol intake and all-cause mortality [Hazard Ratio (HR): 0.73 (95% Confidence Interval, 0.59-0.91)]. Compared to non-drinkers, light (< 5 g alcohol/day) and moderate (5-15 g alcohol/day) drinking of total wine, red wine, and beer was inversely associated with all-cause mortality, with HRs of 0.70 (0.56-0.88), 0.62 (0.49-0.78), and 0.62 (0.67-0.84), respectively. Wine was associated with a lower incidence of cardiovascular events at moderate and high (>15-70 g alcohol/day) consumption levels, with HRs of 0.73 (0.51-0.99) and 0.29 (0.18-0.47), respectively. Light and moderate beer consumption was also associated with a lower incidence of cardiovascular events [HR: 0.75 (0.62-0.88)]. Alcohol intake was associated with reduced cardiovascular and cancer mortality in participants assigned the Mediterranean diet, but not in those allocated the control, low-fat diet.

Conclusions and Relevance: In an older Mediterranean population at high cardiovascular risk, light and moderate consumption of alcoholic beverages, mainly red wine and beer, was inversely associated with total mortality and major cardiovascular events.
Trial Registration: ISRCTN of London, England: 35739639. http://www.isrctn.com/ISRCTN35739639.

KEYWORDS: beer, cardiovascular disease, mortality, myocardial infarction, stroke, wine.

ABBREVIATIONS:

AMI, acute myocardial infarction; FFQ, food frequency questionnaire; CHD, coronary heart diseases; MedDiet, Mediterranean diet; ORW, old red wine; RW, red wine; WW, white wine; YRW, young red wine.

INTRODUCTION

Although excessive alcohol consumption is unquestionably harmful, consistent epidemiological evidence suggests that moderate alcohol consumption is inversely associated with cardiovascular risk factors and cardiovascular disease events, independently of the beverage consumed[1–3]. Nevertheless, current studies suggest that fermented alcoholic beverages may confer greater protective effects than distilled beverages[4,5], possibly because of their higher polyphenolic content[1–3]. In fact, recent data show that the non-alcoholic fraction of red wine (RW), mainly polyphenols, decreases blood pressure[6], improves glucose metabolism[7], and reduces the expression of leukocyte and endothelial adhesion molecules related to atherosclerosis[8], all known cardiovascular risk factors. Likewise, the non-alcoholic fraction of beer was also shown to maintain endothelial integrity[9] and reduce plasma inflammatory biomarkers[9].

Most studies that have evaluated moderate alcohol consumption in relation to health effects are retrospective and as such are limited by possible reverse causality and unmeasured confounders[5]. We carried out a prospective study aimed at assessing differential associations of wine, beer and spirit consumption with all-cause mortality and incidence of cardiovascular events within the context of the PREDIMED (PREvención con DIeta MEDiterránea) trial.

SUBJECTS AND METHODS

Study design and population

The present study is an observational prospective cohort analysis within the PREDIMED trial. This study was a large, multicenter, randomized, parallel group and controlled trial aimed at assessing the effects of two Mediterranean diets (MedDiet), supplemented with

either extra virgin olive oil or mixed nuts, versus a low-fat control diet on cardiovascular outcomes in individuals at high cardiovascular risk. Details of the recruitment method and study design have been described[10] and are available at www.predimed.es. Participants who reported excessive alcohol intake in the CAGE questionnaire (a four-item screening tool)[11] were not eligible for the trial. The trial was scheduled for a duration of 6 years, but was stopped after 4.8 years of follow-up due to early evidence of a cardio-protective effect of the MedDiets[12].

This study was conducted according to the guidelines laid down in the Declaration of Helsinki. All participants provided written informed consent and all procedures involving human subjects/patients were approved by the Institutional Review Boards of the participating centers (Clinical Trial Registration: ISRCTN of London, England: 35739639, http://www.isrctn.com/ISRCTN35739639).

From the total of 7447 recruited participants, we excluded 78 participants with missing dietary data, 149 participants due to extremes of total energy intake (>4,000 or <800 kcal per day in men and >3,500 or <500 kcal per day in women), and 66 participants with alcohol intake higher than 70 g/day. Thus, data from 7154 participants were available for analyses.

Measurements

Assessment of diet, alcohol intake and covariates

Consumption of alcoholic beverages and other dietary variables was assessed at baseline and yearly using repeated measures of a 137-item food frequency questionnaire (FFQ), previously validated in Spain[13,14]. The FFQ included seven questions on the amount and frequency of consumption of different alcoholic beverages (wine, red wine, young red wine, old red wine, white wine, beer and spirits). The intra-class correlation coefficient between alcohol intake

from the FFQ and repeated food records was 0.82[13]. Total energy and nutrient intake were estimated by using Spanish food composition tables[15].

At baseline and yearly, participants were also asked to filled out a questionnaire about adherence to the traditional MedDiet, lifestyle, socio-demographic characteristics, concurrent diseases, and medication used, as well as a validated version of the Minnesota Leisure Time Physical Activity Questionnaire[16].

Categories of alcohol intake

We calculated the updated cumulative average of alcohol intake using repeated measurements of diet from all available FFQs. When alcohol intake values were missing, the most recent available FFQ was used and corrected by the last observation carry forward method. Participants were divided into four groups according to their alcohol intake level: non-drinkers (0 g/d), from >0 to <5 g/d (light drinkers), from 5 to 15 g/d (moderate drinkers), and higher than 15 g/d but lower than 70 g/d (high drinkers). The same groups were used to assess the specific associations of wine [total, red (RW), young red (YRW), old red (ORW) and white wine (WW)], beer, and spirits. In order to calculate the cumulative intake in equivalent g of alcohol/day, we used the average alcohol content of each beverage as 12% alcohol for wine, 5% for beer, and 40% for spirits.

Assessment of outcomes

The main endpoint was the occurrence of major cardiovascular events. These comprised acute myocardial infarction (AMI), stroke and death due to cardiovascular causes. Additionally, we also repeated analyses using death from any cause as outcome. Stroke was further categorized according to the Nation Survey of Stroke Criteria[17] into ischemic (embolic or thrombotic) and hemorrhagic strokes (subarachnoid or intracerebral). Mortality was ascertained through contacts with participants, family physicians, review of medical records, and linkage to the National Death Index. All outcomes were reported between

October 1, 2003 and December 1, 2010. A review of all medical records of participants was completed yearly in each center by physician-investigators who were blinded to the intervention. All outcomes were reported to the event adjudication committee, whose members were blinded to treatment allocation. The committee confirmed major events, determined the cause of death and updated information on these endpoints once per year during the trial[10].

Statistical analyses

We calculated years of follow-up for each individual from the date of inclusion to the study to the date of the primary event, death, or the end of follow-up, whichever came first. Baseline characteristics are presented as means (±SD) for continuous variables and frequencies for categorical variables across groups of total alcohol intake. Differences between groups were tested by a one-factor ANOVA for continuous variables and by the "χ^2 test" for categorical variables. Hazard ratios estimated from time-dependent Cox proportional hazard regression models were used to evaluate the prospective associations between alcohol intake and incidence of major cardiovascular events or death, using the non-drinkers group as the reference category. We tested the homogeneity of the associations by pre-specified strata (sex, age, baseline diabetes, smoking status, family history of early coronary heart diseases (CHD), and dietary intervention group) to evaluate potential heterogeneity in the associations. We carried out stratified analysis in the pre-specified strata and tested the potential interactions in the multivariate models. To test for statistical interactions, we added to the model the interaction product between total alcohol intake or intake of the alcoholic beverage (both as categorical variable) and the corresponding variable studied for interaction. Likewise, we also analyzed specific death risk (cardiovascular death, cancer death, or death from other causes) by total alcohol intake and dietary intervention group. In addition, we assessed the association between alcohol intake and the risk of death or cardiovascular events

according to the alcoholic beverage preference. Participants reporting wine or beer intake > 75% of alcohol were considered as wine or beer drinkers, respectively.

Overall, we used three different models based on the following covariates: 1) unadjusted model; 2) model 1 with adjustment for age, physical activity, total energy intake, baseline body mass index, baseline waist circumference, smoking status (never, current or former smokers categorized in three groups: no smoking since 0 to 1 year, 1 to 5 years, more than 5 years), and intake of wine, beer or spirits, depending on the type of alcohol; and 3) model 2, additionally adjusted for baseline prevalence of hypertension, dyslipidemia, and diabetes; use of aspirin, antihypertensive drugs, hypolipidemic drugs, oral hypoglycemic agents, insulin, and vitamins; education (in two categories: primary school and high school plus university studies); intake of protein, saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, and cholesterol. We adjusted all models by sex, intervention group, and recruitment center. Furthermore, we evaluated the non-linear association between wine and beer intake and incident cardiovascular events and all-cause mortality by restricted cubic splines. Statistical analyses were conducted in RStudio software version 0.99.446 (R software v 3.1.1) using the Survival R package v. 2.37-7 and rms R package v. 4.3-1 to perform the survival analysis and the restricted cubic spline model. All tests were 2-sided and *P*-values <0.05 were considered statistically significant.

RESULTS

Baseline characteristics

The baseline characteristics of participants (n=7154) according to their total alcohol intake are shown in **Table 1**. Participants with a higher consumption of alcohol were generally men, more physically active, less obese and displayed greater adherence to the Mediterranean diet than non-drinkers. High alcohol consumption was also associated with a greater

prevalence of smoking and a lower prevalence of hypertension, diabetes, and dyslipidemia. Accordingly, participants with a high daily alcohol intake took fewer medications (antihypertensive, hypolipidemic, and hypoglycemic drugs). The most consumed alcoholic beverage was wine (mean (SD): 93.7 (117.4) mL/d in men and 22.8 (51.4) mL/d in women), followed by beer (73.2 (166.6) mL/d in men and 16.7 (60.7) mL/d in women).

Alcohol, beverage type and all-cause mortality

During a mean follow-up of 4.8 years and 30,786 person-years, a total of 322 deaths occurred. In this period, 272 cardiovascular events were also diagnosed: 102 AMI (12 fatal), 132 strokes (108 ischemic), and 79 cardiovascular deaths.

The Cox regression analysis using groups of yearly-updated cumulative intake of alcohol showed an inverse association between alcohol consumption and all-cause mortality (**Table 2**). In the multivariable-adjusted model 2, light and moderate consumption of alcohol as wine, RW, beer and total alcohol was inversely associated with all-cause mortality with a relative risk reduction of 30% for wine, 38% for RW and beer, and 39% for total alcohol. Unstable associations were observed at high consumptions of wine, RW and beer (>15-70 g alcohol/day). An increased risk of all-cause mortality was observed in the unadjusted model, whereas non-significant associations were detected in the multivariable adjusted model 2 (except for the inverse association detected in young red wine). ORW, WW and spirits were only inversely associated with all-cause mortality in the lower consumption group, with a reduced risk of 48%, 47%, and 46%, respectively. Total wine consumption significantly reduced all-cause mortality risk by 57%, 51% and 39% at >0-5, 5-15 and >15-70 g alcohol/d, respectively (**eTable 4** in supplemental material).

Figure 1 exhibits the J-shaped curve, which provides evidence for the inverse association against all-cause mortality of wine and beer at low and moderate levels of consumption (<20 g alcohol/d), but a direct association at higher levels of consumption. Wine

showed the strongest inverse association with all-cause mortality, followed by beer **(Table 2)**. However, both beverages increase the relative risk of all-cause mortality when consumed at levels >25 g alcohol/d.

Alcohol, beverage type and incident cardiovascular events

Table 3 shows hazard ratios for total incident cardiovascular events and by ischemic stroke, myocardial infarction, and cardiovascular death. Alcohol intake was associated with a significantly lower incidence of cardiovascular events, with a reduction of 47% in the highest group of alcohol intake (>15 g alcohol/d). At moderate-high consumption levels relative risk reduction was 71% for total wine, 38% for RW, and 34% for YRW. Beer consumption was significantly associated with a 27% decrease in the relative risk of incident cardiovascular events in the low and moderate groups (>0-5 and >5-15 g alcohol/d). However, the daily consumption of more than 15 g alcohol as beer was significantly associated with a 71% increase of incident cardiovascular events.

Figure 2 shows the J-shaped association between the type of alcoholic beverage (beer and wine) consumption and cardiovascular events. Low consumption of beer reduced the risk of cardiovascular events, but the association was not significant. Wine showed the strongest protective effect against cardiovascular events at low, moderate ,and high consumption levels.

Heterogeneity Analyses

All-cause mortality

Table 4 and **eTable 1** show the stratified analysis of all-cause mortality for total alcohol intake and for wine and beer intake, respectively. Sex, diabetes, smoking status and dietary intervention group showed a significant interaction. Thus, the association between alcohol intake and all-cause mortality was much greater in men and remained statistically significant at higher consumption (>15 g alcohol/d for wine and total alcohol, and >5-15 g alcohol/d for

beer). After adjusting for confounders, we found a 51% decreased relative risk of all-cause mortality compared to the reference group among men in the top group of total alcohol consumption. However, when all participants (men and women) were included in the model, a non-significant reduction of 22% was found. The inverse association of total alcohol intake with all-cause mortality was also greater in diabetics and smokers.

Interestingly, the dietary intervention group had also a powerful effect in the model. Thus, the combination of alcohol intake (either wine or beer) and allocation to a Mediterranean diet intervention was associated with a reduced risk of 41% (at >15 g alcohol/day) for all-cause mortality. However, alcohol intake in the low-fat diet (control) intervention group had no significant association with all cause-mortality at levels >0-5 and >5-15 g alcohol/d and increased the risk by 96% at higher levels (>15 g alcohol/d).

When we stratified by the type of death (cardiovascular, cancer or other causes) according to diet and alcohol intake (**Table 5 and eTable 2**), cancer death and death from other causes showed a significant interaction in the model. The low-fat control diet in combination with alcohol intake more than tripled the risk of death from cancer (increased risk of 332%) at > 15 g alcohol/d. By contrast, the combination of Mediterranean diet and alcohol intake had no significant association with cancer death. However, when the type of alcoholic beverages was taken into account, the results were different. The combination of wine consumption and a Mediterranean diet reduced the risk of death from cancer by 42% and 30% at doses of >5-15 and >15 g alcohol/d, respectively, whereas the combination of wine and a low-fat diet increased the risk death from cancer by 3.7, 3.1 and 6 times at >0-5, >5-15 and >15 g alcohol/d, respectively.

Finally, alcohol intake in the low-fat diet group was not significantly associated with death from other causes, while the combination of Mediterranean diet and alcohol intake decreased the risk of death from other causes by 65% at intake > 15 g alcohol/d. In respect to

wine, its consumption with the Mediterranean diet was associated with a reduced the risk of death from other causes, whereas its consumption with a low-fat diet increased the risk of death from other causes.

Incident cardiovascular events

The stratified analyses for cardiovascular events (**Table 6 and eTable 3**) showed that smoking, family history of CHD, and diet also had significant interactions in the model. Alcohol intake was inversely associated with the risk of any cardiovascular event in subjects who were non-smokers, non obese and had no family history of CHD. The combination of a Mediterranean diet and consumption of > 15 g alcohol/d was also significantly associated with a reduced risk of cardiovascular events. However, alcohol intake in the low-fat diet group was not significantly associated with the risk of incident cardiovascular events. The analysis using only wine drinkers (**eTable 3**) shows similar results for wine consumption, which significantly reduced the incidence of cardiovascular events by 47% at >15 g alcohol/d in the Mediterranean diet group, but showed no significant association in the control group.

Alcohol, beverage type and incident stroke

Wine, RW, YRW were associated with a reduction in the risk of ischemic stroke by 43%, 43%, and 37%, respectively, in the >5-15 g alcohol/d consumption group (**Table 3**). Beer was also associated with a decrease in ischemic stroke, although the association was weaker and it was only observed in the low consumption group (>0-5 g alcohol/day) with a 32% reduction. At intakes > 15 g alcohol/day of beer, the risk of ischemic stroke increased significantly by 126%. The low number of hemorrhagic strokes (n=25) precluded studying their association with alcohol intake.

Alcohol, beverage type and incident myocardial infarction

AMI was inversely associated with low, moderate and high (>15 alcohol/d) consumption of wine, RW, and YRW. The relative risk of AMI was reduced by 70%, 65%, and 71%,

respectively (**Table 3**). Beer drinking reduced the risk of AMI in the >5-15 g alcohol/day consumption group by 44%.

Alcohol, beverage type and cardiovascular death

Total alcohol intake was not associated with cardiovascular death (**Table 3**). However, when the analysis was performed by beverage type, low consumption of wine (>0-5 g alcohol/day) was significantly associated with a reduced cardiovascular death risk of 47%, moderate consumption of RW and YRW (>5-15 g alcohol/d) reduced risk by 14% and 50%, and low consumption of beer (>0-5 g alcohol/d) by 53%.

DISCUSSION

In this pre-specified analysis of the PREDIMED trial cohort considered as a prospective study, asymptomatic subjects at high cardiovascular risk consuming low and moderate amounts of wine and beer had a 38% reduction in the risk of all-cause mortality after a mean follow-up of 4.8 years. Wine drinking at moderate and high levels was associated with a 71% reduction of cardiovascular disease events, while beer at low and moderate intake reduced cardiovascular events by 27%, but increased risk by 71% at high doses (>15 g of alcohol/d). Importantly, the effects of alcohol intake (either wine or beer) varied depending on the dietary pattern. Alcohol intake at >15 g/d in subjects assigned to the Mediterranean diet intervention was associated with a 41% reduced risk of all-cause mortality. By contrast, alcohol intake by those allocated to the low-fat diet intervention had no significant effects on all cause-mortality at levels of >0-5 and >5-15 g/d, but increased risk by 96% at higher levels (>15 g /day).

Several studies have reported J or U-shaped curves for the relationship between moderate alcohol intake and cardiovascular diseases (CVD) and total mortality. The increased mortality risk in non-drinkers observed in our study is consistent with the J-shaped

dose-response relationship widely reported in observational studies[25-27]. In general, the specific type of alcoholic beverage consumed appears to be less important than the consistency with which alcohol is consumed. The beverage most likely to be inversely related to cardiovascular disease in a given population tends to be the most widely consumed one in that population[51]. Thus, some systematic reviews have concluded that beverage type is relatively unimportant[52]. However, two other recent meta-analyses that evaluated different alcoholic beverages reached a different conclusion. One indicated that low-to-moderate wine and beer consumption was indeed associated with a decrease in cardiovascular events and mortality[19], and the other concluded that consumption of wine, but not beer or spirits, was inversely associated with cardiovascular risk[20]. The results of the current study were in the line with those of these last meta-analyses.

In our study, alcohol intake was also associated with a reduced risk of ischemic stroke. In a meta-analysis, moderate alcohol intake had little or no association with the risk of total stroke, hemorrhagic stroke, ischemic stroke, and stroke mortality[21], whereas Reynolds et al.[22] observed that alcohol consumption of less than 24 g/d was associated with a reduced risk of ischemic stroke, whereas intake of more than 60 g/d was associated with an increased risk of total stroke and its subtypes. Another meta-analysis suggested that heavy alcohol consumption increased the risk of any stroke but light or moderate alcohol consumption protected against ischemic stroke[23]. With respect to the type of alcoholic beverages, some cohort studies such as the Zutphen Study[24] and a meta-analysis[31] reported that long-term low-to-moderate consumption of wine and beer was inversely associated with cerebrovascular disease, with a highest effect at ≤20 g of ethanol/d. The effects of spirits were different[25]. Similar to these results, we observed that wine and beer drinkers had a significantly lower incidence of cardiovascular events, however consumption of beer at > 15 g alcohol/d was significantly associated with an increase of cardiovascular events.

Women are more sensitive to the toxic effects of alcohol than men[26]; thus, the recommended upper limit of alcohol consumption for women is one-half of that recommended for men[27]. Our results confirm the increase risk of all-cause mortality when alcohol intake was ≥4 drinks/day in men and ≥2 drinks/day in women, as shown by Di Castelnuovo et al.[18]

Despite the large body of scientific evidence on the health benefits of moderate alcohol consumption[48], the mechanisms implicated are still not completely known. There is consistent evidence that low and moderate intake of alcohol increases plasma HDL cholesterol, enhances insulin sensitivity, and improves endothelial function and hemostasis[4]. Beyond their alcohol content, wine and beer posses antioxidant and anti-inflammatory properties and have a beneficial effect on the vascular endothelium via stimulation of nitric oxide synthesis[5,6]. Part of these effects might be related to the higher polyphenolic content of wine and beer compared to other alcoholic beverages[28–31]. Thus, polyphenols and their metabolites may provide additional benefits on CVD risk by decreasing blood pressure, inhibiting LDL oxidation, increasing nitric oxide release, improving endothelial function and reducing inflammation and cell adhesion molecules[32–34].

The major polyphenols in beer are hydroxybenzoic acids, cinnamic acids such as ferulic acid, and flavonols like kaempferol and quercetin glucosides[35]. The major polyphenols in RW are anthocyanindins such as malvidin-3-*O*-glucoside, procyanidins, tannins, flavanols like catechin and epicatechin, and benzoic acids such as gallic acid. During wine aging the phenolic profile changes, since anthocyanidins and tannins undergo various degradation processes[36,37]. Phenol losses with wine aging may explain why YRW consumption had a stronger association with reduced all-cause mortality, AMI and cardiovascular death than ORW. In addition, the different phenolic composition of wine and beer may also explain the differences observed in their effects on CVD.

Another relevant finding from our study is that the association of alcohol intake with all-cause mortality and incidence of cardiovascular events was modulated by diet. Thus, the combination of allocation to the Mediterranean diet and moderate intake of alcohol (as wine or beer) was inversely associated with all-cause mortality, in particular cancer death and death from other causes. Moderate to high alcohol intake has been associated with a higher incidence of some cancers, such as breast, colorectal, oral cavity, pharynx, larynx, liver, and esophagus cancer[47]. However, according to our results, adherence to the Mediterranean diet may reverse the association of cancer and alcohol.

Our study has limitations. First, given the characteristics of our cohort, the results may be only valid for old people and cannot be extrapolated to other populations. Second, although we controlled for many potential confounders in multivariate models, other unknown confounders related to a healthy lifestyle may exist. On the other hand, the current study also has several strengths, such as the prospective design, large sample size, relative long follow-up, and extensive data on risk factors and confounders. Moreover, we used cumulative average intake of alcohol and beverage type across yearly repeated measures, since it is reported to be the best approach to reduce measurement error in nutritional epidemiology[49,50].

In conclusion, we have observed that a moderate consumption of alcoholic beverages (wine and beer) is inversely associated with total mortality and the risk of cardiovascular events. However, the protective effects of wine, especially young red wine, seems to be higher than those of beer, and, more interestingly, these effects varied depending on the dietary pattern followed by the participants. The healthier association was the combination of Mediterranean diet and moderate wine consumption.

ACKNOWLEDGEMENTS

We thank all the volunteers involved in the PREDIMED study for their valuable cooperation. We also would like to thank to Oriol Pages-Figueras for his kindly help in the figures preparation. CIBEROBN is an initiative of ISCIII, Spain.

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Sources of funding and support: This work was supported in part by FIS 13-02184 and INNPRONTA from the Instituto de Salud Carlos III (ISCIII), CICYT (AGL2010-22319-C03; AGL2013-49083-C3-1-R) from the Spanish Ministry of Science and Innovation (MICINN), the European Foundation for Alcohol Research (ERAB) EA 1117 and EA 1324 and Quality

Group from Generalitat de Catalunya Generalitat de Catalunya (GC) 2014 SGR 773. The CIBEROBN is an initiative of the ISCIII, Spain. P.Q. R is grateful for the predoctoral fellowships awarded by the Generalitat de Catalunya (FI-DRG). A. M.-R. thanks the "Juan de la Cierva" postdoctoral program (JCI-2012-13463) from MEC (Ministerio de Economía y Competitividad). The funding source had no role in the trial design, data analysis, reporting of the results, or decision to submit the manuscript for publication.

Disclosure of conflict of interest: Dr. Estruch reports serving on the board of and receiving lecture fees from the Research Foundation on Wine and Nutrition (FIVIN); serving on the boards of the Beer and Health Foundation and the European Foundation for Alcohol Research (ERAB); receiving lecture fees from Cerveceros de España and Sanofi-Aventis; and receiving grant support through his institution from Novartis. Dr. Lamuela-Raventos reports serving on the board of and receiving lecture fees from FIVIN; receiving lecture fees from Cerveceros de España; and receiving lecture fees and travel support from PepsiCo. Dr. Salas-Salvadó reports serving on the board of and receiving grant support through his institution from the International Nut and Dried Fruit Council; receiving consulting fees from Danone; and receiving grant support through his institution from Eroski and Nestlé. Dr. Ros reports grants, non-financial support and other from California Walnut Commission, grants, personal fees, non-financial support and other from Merck, Sharp & Dohme, grants, personal fees, non-financial support and other from Alexion, personal fees, non-financial support and other from Aegerion, grants and personal fees from Sanofi Aventis, grants, personal fees, non-financial support and other from Ferrer International, grants from Amgen, grants from Pfizer, personal fees from Akcea, outside the submitted work. Dr. Arós reports receiving payment for the development of educational presentations from Menarini and AstraZeneca. Dr. Pintó reports serving on the board of and receiving grant support through his institution from the Residual Risk Reduction Initiative (R3i) Foundation; serving on the board of Omegafort;

serving on the board of and receiving payment for the development of educational presentations, as well as grant support through his institution, from Ferrer; receiving consulting fees from Abbott Laboratories; receiving lecture fees, as well as grant support through his institution, from Merck and Roche; receiving lecture fees from Danone and Esteve; receiving payment for the development of educational presentations from Menarini; and receiving grant support through his institution from Sanofi-Aventis, Kowa, Unilever, Boehringer Ingelheim, and Karo Bio. Nevertheless, these foundations had no involvement in the study design, the collection, analysis and interpretation of data, the writing of the manuscript or the decision to submit the manuscript for publication.

The other authors declare no conflict of interest.

Dr. Estruch, Dr. Corella and Paola Quifer-Rada had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis

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FIGURES

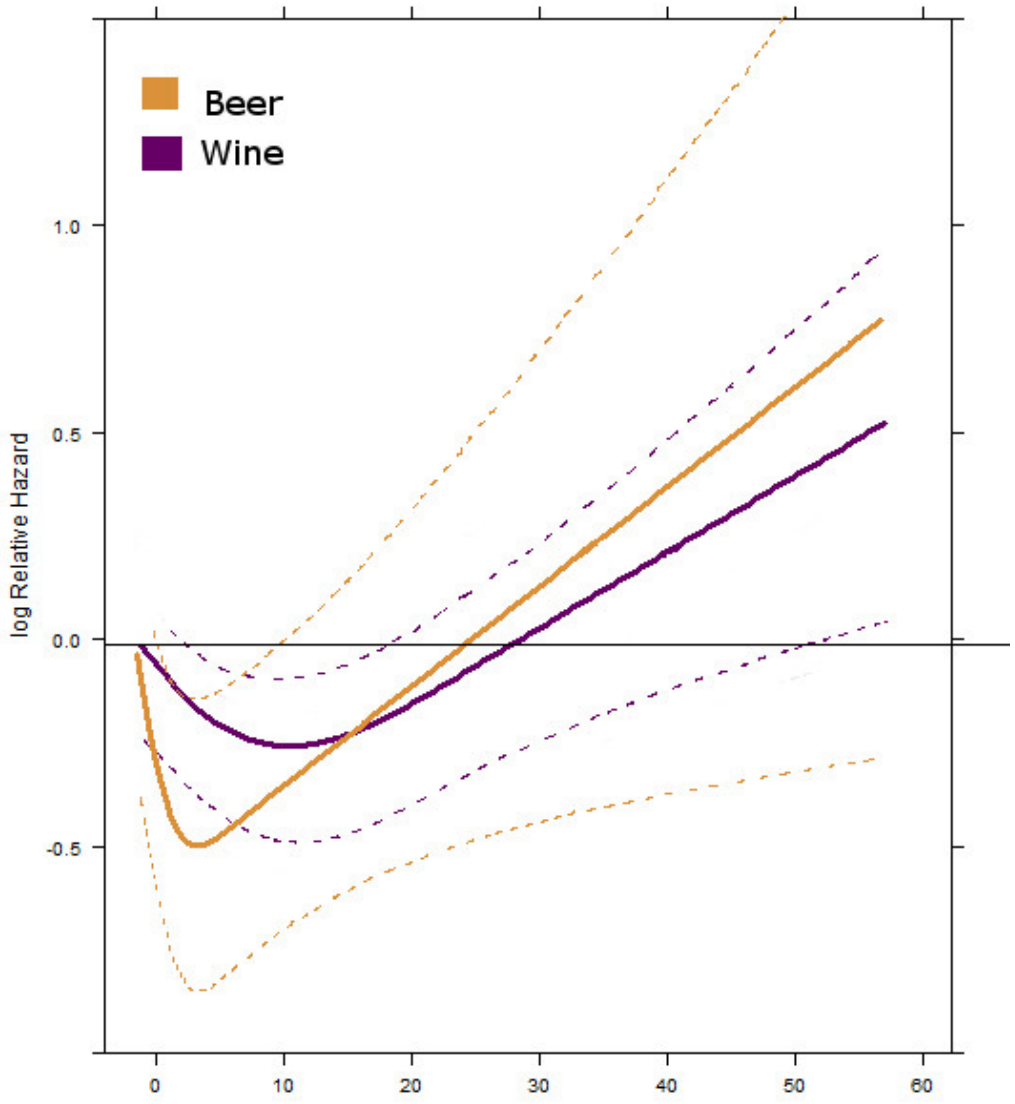

Figure 1. Relative hazard (logarithmic scale) of all-cause mortality by average cumulative consumption of beer and wine (g/d). Restricted cubic spline model adjusted by age (continuous variable), physical activity, total energy intake, baseline body mass index, baseline waist circumference, smoking status (never, former or current smoker), intake of beer or wine (beer model was adjusted by wine intake and wine model was adjusted by beer intake) and liquors (only in the beer, wine and red wine model), recruitment center, sex, intervention group, hypertension at baseline, dyslipidemia at baseline, diabetes at baseline, use of aspirin, antihypertensive drug, hypolipidemic drugs, oral hypoglycaemic agents, insulin, and vitamins intake; intake of protein, saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, cholesterol.

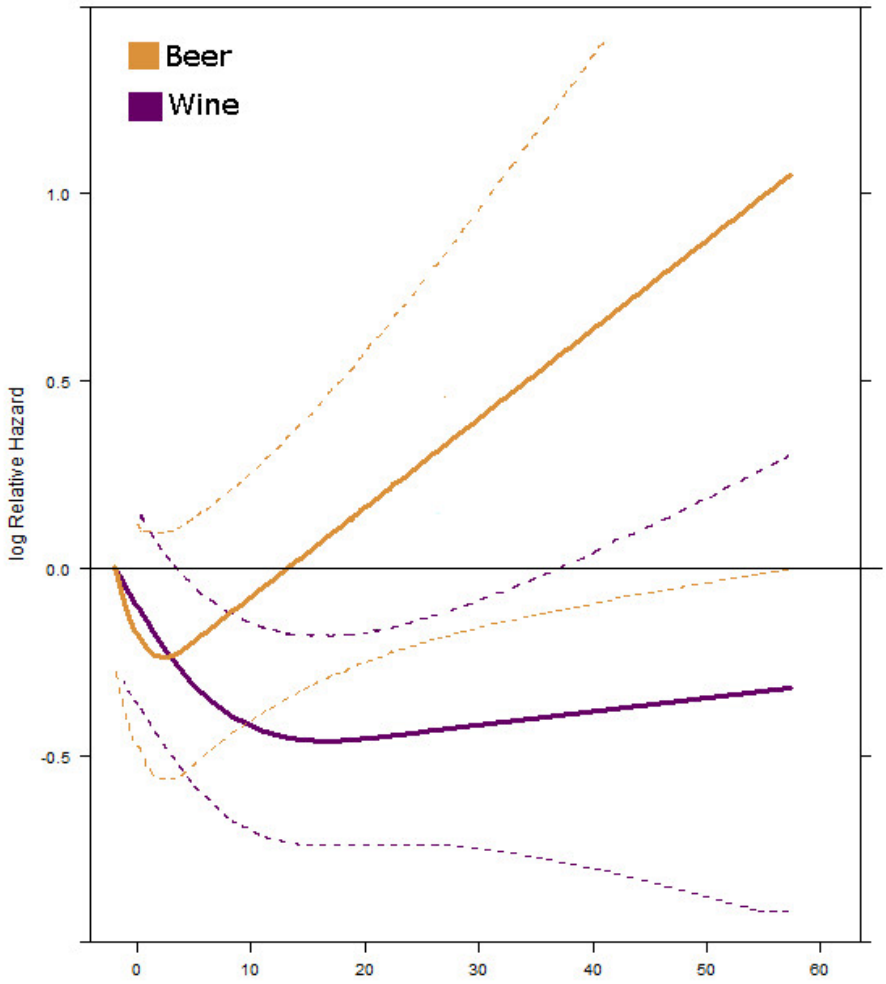

Figure 2. Relative hazard (logarithmic scale) of cardiovascular events by average cumulative consumption of alcohol (g/d). Restricted cubic spline model adjusted by age (continuous variable), physical activity, total energy intake, baseline body mass index, baseline waist circumference, smoking status (never, former or current smoker), intake of beer or wine (beer model was adjusted by wine intake and wine model was adjusted by beer intake) and liquors (only in the beer, wine and red wine model), recruitment center, sex, intervention group, hypertension at baseline, dyslipidemia at baseline, diabetes at baseline, use of aspirin, antihypertensive drug, hypolipidemic drugs, oral hypoglycaemic agents, insulin, and vitamins intake; intake of protein, saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, cholesterol.

TABLES

Table 1. Baseline characteristics of the study participants according to groups of total alcohol intake (g/day).

	0	**>0-5**	**>5-15**	**>15-70**	***P****
No. of subjects	2650	1788	1572	1144	
Age (y), mean (SD)	68.4 (5.9)	67 (5.9)	66.5(6.1)	65.5 (6.0)	<0.001
Women, n (%)	2151 (81.1)	1149 (64.2)	684 (43.5)	162 (14.1)	<0.001
BMI (kg/m^2), mean (SD)	30.3 (4.1)	30.2 (3.9)	29.5 (3.6)	29.2 (3.2)	<0.001
Waist circumference (cm), mean (SD)	98.9 (10.8)	99.8 (10.2)	100 (10)	101.8 (9.1)	<0.001
Weight (kg), mean (SD)	73.4 (11.3)	75.9 (11.7)	77.3 (12)	80.3 (11)	<0.001
Waist to height ratio, mean (SD)	0.63 (0.07)	0.63 (0.06)	0.62 (0.06)	0.61 (0.05)	<0.001
Hypertension, n (%)	2259 (85.2)	1492 (83.4)	1264 (80.4)	905 (79.1)	<0.001
Diabetes, n (%)	1374 (51.8)	888 (49.6)	713 (45.3)	525 (45.9)	<0.001
Dyslipidemia, n (%)	1925 (72.6)	1309 (73.2)	1130 (71.8)	842(70.1)	0.29
Current smoker, n (%)	192 (7.2)	202 (11.3)	255 (16.2)	328 (28.6)	<0.001
Family history of premature CHD, n (%)	608 (22.9)	432 (24.1)	327 (20.8)	238 (20.8)	0.10
Medication:					
Antihypertensive drugs, n (%)	2020 (76.3)	1312 (73.4)	1054 (67.1)	772 (67.5)	<0.001
Statins (hypolipidemic drugs), n (%)	1363 (51.4)	888 (49.7)	729 (46.3)	540 (47.2)	0.006
Insulin, n (%)	181 (6.8)	99 (5.5)	53 (3.3)	40 (3.5)	<0.001
Oral hypoglycemic drugs, n (%)	943 (35.6)	597 (33.3)	438 (27.9)	326 (28.5)	<0.001
Aspirin or other antiplatelet agents, n (%)	594 (22.4)	413 (23.1)	331 (21)	263 (22.9)	0.7
Vitamins or supplements, n (%)	384 (14.5)	204 (11.4)	138 (8.7)	57 (4.9)	<0.001
Educational level:					<0.001
Primary school, n (%)	2159 (81.5)	1299 (72.6)	1092 (69.4)	703 (61.4)	
High school, n (%)	244 (9.2)	276 (15.4)	296 (18.8)	268 (23.4)	
Graduate, n (%)	91 (3.6)	131 (7.3)	137 (8.7)	146 (12.7)	
Nutrient intake and physical activity:					
Total energy intake, mean (SD), Kcal/d	2064 (511)	2181 (504)	2325 (509)	2554 (528)	<0.001
Carbohydrates, mean (SD), g/d	225.6 (72.7)	231.1 (70.2)	241.9 (73.7)	248.8 (76.2)	<0.001
Protein, mean (SD), g/d	87.2 (21.1)	92.3 (20.9)	93.9 (21.0)	95.6 (21.5)	<0.001

Total fat, mean (SD), g/d	90.4 (27.4)	97.0 (28)	101.5 (27.4)	106.4 (27.8)	<0.001
SFA, mean (SD), g/d	27.4 (8.1)	25.1 (8.2)	26.1 (8.3)	27.4 (8.7)	<0.001
MUFA, mean (SD), g/d	44.7 (14.9)	48.0 (15.2)	50.3 (14.5)	53.1 (14.9)	<0.001
PUFA, mean (SD), g/d	14.5 (6.4)	15.4 (6.7)	16.2 (6.4)	17.2 (6.5)	<0.001
Fiber, mean (SD), g/d	25.1 (8.8)	25.4 (8.7)	25.4 (8.6)	25.1 (8.1)	0.50
Cholesterol, mean (SD), mg/d	332 (124)	370 (123)	378 (118)	393 (123)	<0.001
Alcohol, mean (SD), g/d	0	2.0 (1.4)	9.8 (2.6)	31.2 (11.5)	<0.001
Wine, mean (SD), g alcohol/d	0	1.1 (1.3)	6.6 (3.4)	20.9 (11.6)	<0.001
Beer, mean (SD), g alcohol/d	0	0.7 (1.2)	2.3 (3.5)	5.9 (10.0)	<0.001
Spirits, mean (SD), g alcohol/d	0	0.1 (0.3)	0.4 (1.0)	2.5 (4.7)	<0.001
14-point MedDiet score, mean (SD)	8.4 (1.9)	8.5 (1.8)	8.9 (1.9)	9.1 (1.9)	<0.001
Energy expenditure in physical activity (METs-min/day), mean (SD)	222 (200)	250 (229)	283 (264)	328 (263)	<0.001

*One-factor ANOVA was used for continuous variables and χ^2-test for categorical variables.
SD: Standard Deviation
BMI: Body mass index
CHD: Coronary heart disease
SFA: Saturated fatty acids
MUFA: Monounsaturated fatty acids
PUFA: Polyunsaturated fatty acids
MedDiet: Mediterranean diet

Table 2. Cox proportional hazard ratios for total mortality according to categories of cumulative average of daily alcohol intake in the PREDIMED study.

Groups according to alcohol intake (g/d)	**0**	**>0-5**	**>5-15**	**>15-70**	***p- trend***	
					Lineal	***square***
Total alcohol, mean (g alcohol/d)	0	1.6	9.5	28.0		
No. of deaths	115	81	55	71		
No. of person-years	11408	7569	6657	5150		
Unadjusted HR (95% CI)	1.00 (ref.)	0.69(0.58-0.83)	0.81(0.67-0.99)	1.28(1.07-1.53)	0.001	<0.001
Multivariable-adjusted 1 HR (95% CI)[1]	1.00 (ref.)	0.66(0.53-0.82)	0.61(0.48-0.77)	0.71(0.56-0.91)	0.83	<0.001
Multivariable-adjusted 2 HR (95% CI)[5]	1.00 (ref.)	0.73(0.59-0.91)	0.68(0.54-0.87)	0.78(0.60-1.01)	0.982	<0.001
Wine, mean (g alcohol/d)	0	1.7	9.1	24.6		
No. of deaths	146	70	55	51		
No. of person-years	14621	7471	5050	3643		
Unadjusted HR (95% CI)	1.00 (ref.)	0.70(0.59-0.83)	0.83(0.69-1.00)	1.35(1.13-1.63)	<0.001	<0.001
Multivariable-adjusted 1 HR (95% CI)[1,2,4]	1.00 (ref.)	0.68(0.56-0.83)	0.61(0.49-0.77)	0.79(0.63-1.0)	0.794	<0.001
Multivariable-adjusted 2 HR (95% CI)[5,2,4]	1.00 (ref.)	0.75(0.61-0.92)	0.70(0.56-0.88)	0.85(0.67-1.08)	0.96	0.001
Red Wine, mean (g alcohol/day)	0	1.7	9.0	24.2		
No. of deaths	175	56	48	43		
No. of person-years	16553	6737	4362	3132		
Unadjusted HR (95% CI)	1.00 (ref.)	0.57(0.48-0.67)	0.72(0.59-0.87)	1.26(1.05-1.52)	0.001	<0.001
Multivariable-adjusted 1 HR (95% CI)[1,2,4]	1.00 (ref.)	0.59(0.48-0.72)	0.53(0.43-0.67)	0.78(0.62-0.98)	0.628	<0.001
Multivariable-adjusted 2 HR (95% CI)[5,2,4]	1.00 (ref.)	0.65(0.53-0.79)	0.62(0.49-0.78)	0.84(0.66-1.06)	0.952	<0.001
Young Red Wine, mean (g alcohol/d)	0	1.69	9.03	24.03		
No. of deaths	215	40	39	28		
No. of person-years	19492	5080	3524	2689		
Unadjusted HR (95% CI)	1.00 (ref.)	0.48(0.41-0.58)	0.69(0.57-0.84)	1.08(0.88-1.33)	0.047	<0.001
Multivariable-adjusted 1 HR (95% CI)[1,2,4]	1.00 (ref.)	0.49(0.40-0.60)	0.53(0.43-0.66)	0.63(0.49-0.80)	0.118	<0.001
Multivariable-adjusted 2 HR (95% CI)[5,2,4]	1.00 (ref.)	0.55(0.45-0.68)	0.62(0.50-0.78)	0.68(0.53-0.88)	0.312	<0.001
Old Red Wine, mean (g alcohol/d)	0	1.22	8.95	23.62		
No. of deaths	273	22	14	13		
No. of person-years	27075	2459	848	403		
Unadjusted HR (95% CI)	1.00 (ref.)	0.54(0.44-0.65)	1.43(1.05-1.95)	1.60(0.94-2.72)	<0.001	<0.001
Multivariable-adjusted 1 HR (95% CI)[1,2,4]	1.00 (ref.)	0.47(0.38-0.59)	1.0(0.73-1.51)	1.08 (0.56-1.86)	<0.001	<0.001
Multivariable-adjusted 2 HR (95% CI)[5,2,4]	1.00 (ref.)	0.52(0.41-0.66)	1.09(0.76-1.58)	1.10(0.56-2.05)	<0.001	<0.001
White Wine, mean (g alcohol/d)	0	0.80	8.82	22.49		
No. of deaths	293	22	2	5		
No. of person-years	27961	2419	265	141		
Unadjusted HR (95% CI)	1.00 (ref.)	0.58(0.46-0.73)	1.60(0.94-2.73)	2.59(1.34-5.01)	<0.001	0.024
Multivariable-adjusted 1 HR (95% CI)[1,2,4]	1.00 (ref.)	0.50(0.38-0.65)	0.74(0.40-1.37)	0.99(0.45-2.2)	0.167	0.015
Multivariable-adjusted 2 HR (95% CI)[5,2,4]	1.00 (ref.)	0.53(0.40-0.70)	0.75(0.40-1.38)	0.98(0.44-2.15)	0.257	0.02
Beer, mean (g alcohol/day)	0	1.23	8.03	26.7		
No.of deaths	225	54	36	7		
No. of person-years	20693	6216	3477	398		

Unadjusted HR (95% CI)	1.00 (ref.)	0.58(0.50-0.68)	0.87(0.67-1.12)	1.40(0.91-2.17)	0.032	<0.001
Multivariable-adjusted 1 HR (95% CI)[1,3,4]	1.00 (ref.)	0.46(0.38-0.55)	0.64(0.47-0.85)	0.96(0.58-1.59)	0.372	<0.001
Multivariable-adjusted 2 HR (95% CI)[5,3,4]	1.00 (ref.)	0.51(0.42-0.61)	0.62(0.46-0.84)	1.01(0.61-1.67)	0.597	<0.001
Spirits, mean (g alcohol/d)	0	1.0	7.6	22.7		
No.of deaths	270	32	20	0		
No. of person-years	25635	3806	1277	68		
Unadjusted HR (95% CI)	1.00 (ref.)	0.71 (0.60-0.86)	2.34 (1.76-3.10)	-	<0.001	0.179
Multivariable-adjusted 1 HR (95% CI)[1,2,3]	1.00 (ref.)	0.50 (0.40-0.62)	1.24 (0.89-1.74)	-	0.001	0.003
Multivariable-adjusted 2 HR (95% CI)[5,2,3]	1.00 (ref.)	0.53 (0.43-0.66)	1.13 (0.80-1.59)	-	0.004	0.009

HR: Hazard ratio; CI: Confidence interval.

[1]The multivariable HR was adjusted by age (continuous variable), physical activity, total energy intake, baseline body mass index, baseline waist circumference, smoking status (never, current or former smokers categorized in three groups: no smoking since 0 to 1 year, 1 to 5 years, more than 5 years), intake of [2]beer, [3]wine or [4]liquors and stratified according to recruitment center, sex, and intervention group.

[5]This model has been additionally adjusted by hypertension at baseline, dyslipidemia at baseline, diabetes at baseline, use of aspirin, antihypertensive drug, hypolipidemic drugs, oral hypoglycaemic agents, insulin, and vitamins intake; education (as two categories: primary school and high school and university studies); intake of protein, saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, cholesterol, [2]beer, [3]wine or [4]liquors (all as continuous variables).

Table 3. Cox proportional hazard ratios for incident cardiovascular events according to categories of cumulative average of daily alcohol intake in the PREDIMED study. Primary end-points are further divided into stroke events, myocardial infarctions and cardiovascular death.

Groups according to alcohol intake (g/d)	**0**	**>0-5**	**>5-15**	**>15-70**	***p-trend***	
					Lineal	***square***
Total alcohol, mean (g/d)	0	1.6	9.5	28.0		
Primary end point						
No. of cases	103	68	54	47		
No. of person-years	11409	7569	6657	5150		
Unadjusted HR (95% CI)	1.00 (ref.)	0.87(0.74-1.03)	1.0(0.84-1.20)	0.90(0.75-1.09)	0.735	0.983
Multivariable-adjusted 1 HR (95% CI)[1]	1.00 (ref.)	0.85(0.70-1.02)	0.74(0.60-0.91)	0.45(0.35-0.58)	<0.001	0.268
Multivariable-adjusted 2 HR (95% CI)[5]	1.00 (ref.)	0.94(0.78-1.14)	0.85(0.69-0.99)	0.53(0.41-0.69)	<0.001	0.20
Ischemic stroke						
No. of cases	45	19	24	20		
Multivariable-adjusted 1 HR (95% CI)[1]	1.00 (ref.)	0.72(0.54-0.96)	0.60(0.43-0.84)	0.49(0.33-0.73)	<0.001	0.278
Multivariable-adjusted 2 HR (95% CI)[5]	1.00 (ref.)	0.87 (0.65-1.16)	0.81 (0.57-1.14)	0.75 (0.49-1.13)	0.024	0.259
Myocardial infarction						
No. of cases	32	32	22	16		
Multivariable-adjusted 1 HR (95% CI)[1]	1.00 (ref.)	1.10(0.80-1.50)	0.74(0.52-1.04)	0.31(0.21-0.48)	<0.001	<0.001
Multivariable-adjusted 2 HR (95% CI)[5]	1.00 (ref.)	1.15(0.84-1.60)	0.79(0.55-1.13)	0.34(0.22-0.52)	<0.001	<0.001
Cardiovascular death						
No. of cases	30	22	10	17		
Multivariable-adjusted 1 HR (95% CI)[1]	1.00 (ref.)	0.63(0.40-0.97)	0.79(0.50-1.23)	0.77(0.47-1.26)	0.893	0.06
Multivariable-adjusted 2 HR (95% CI)[5]	1.00 (ref.)	0.67(0.43-1.06)	0.81(0.52-1.28)	0.87(0.52-1.44)	0.868	0.096
Wine, mean (g/d)	0	1.67	9.09	24.62		
Primary end point						
No. of cases	129	68	42	33		
No. of person-years	14604	7456	5045	3642		
Unadjusted HR (95% CI)	1.00 (ref.)	0.93(0.80-1.08)	0.92(0.77-1.03)	0.94(0.77-1.15)	0.911	0.70
Multivariable-adjusted 1 HR (95% CI)[1,2,4]	1.00 (ref.)	0.92 (0.78- 1.1)	0.65 (0.53-0.80)	0.49(0.38-0.63)	<0.001	0.60
Multivariable-adjusted 2 HR (95% CI)[5,2,4]	1.00 (ref.)	1.22(0.91-1.63)	0.73(0.51-0.99)	0.29(0.18-0.47)	0.01	0.475
Ischemic stroke						
No. of cases	52	24	16	16		
Multivariable-adjusted 1 HR (95% CI)[1,2,4]	1.00 (ref.)	0.88 (0.68-1.15)	0.43(0.29-0.63)	0.64(0.43-0.96)	0.001	0.033
Multivariable-adjusted 2 HR (95% CI)[5,2,4]	1.00 (ref.)	1.05 (0.79-1.38)	0.57 (0.39-0.83)	0.83 (0.55-1.26)	0.111	0.041
Myocardial infarction						
No. of cases	44	32	18	8		
Multivariable-adjusted 1 HR (95% CI)[1,2,4]	1.00 (ref.)	1.12(0.85-1.49)	0.66(0.47-0.92)	0.28(0.18-0.44)	<0.001	<0.001
Multivariable-adjusted 2 HR (95% CI)[5,2,4]	1.00 (ref.)	1.24(0.93-1.65)	0.75(0.53-0.98)	0.30(0.19-0.48)	<0.001	<0.001
Cardiovascular death						
No. of cases	39	17	11	12		
Multivariable-adjusted 1 HR (95% CI)[1,2,4]	1.00 (ref.)	0.50(0.33-0.77)	0.65(0.43-0.99)	0.60(0.37-0.98)	0.347	0.023
Multivariable-adjusted 2 HR (95% CI)[5,2,4]	1.00 (ref.)	0.53(0.35-0.82)	0.73(0.47-1.11)	0.69(0.42-1.12)	0.35	0.02
Red Wine, mean (g/d)	0	1.70	9.03	24.18		
Primary end point						
No. of cases	157	53	33	29		
No. of person-years	16553	6737	4363	3133		
Unadjusted HR (95% CI)	1.00 (ref.)	0.81(0.70-0.94)	0.80(0.67-0.97)	0.97(0.79-1.19)	0.918	0.01
Multivariable-adjusted 1 HR (95% CI)[1,2,4]	1.00 (ref.)	0.79(0.66-0.94)	0.57(0.46-0.70)	0.53(0.41-0.67)	<0.001	0.079
Multivariable-adjusted 2 HR (95% CI)[5,2,4]	1.00 (ref.)	0.86(0.73-1.03)	0.68(0.55-0.85)	0.62(0.48-0.80)	0.012	0.233
Ischemic stroke						
No. of cases	61	20	13	14		

Multivariable-adjusted 1 HR (95% CI)[1,2,4]	1.00 (ref.)	0.80(0.61-1.05)	0.42(0.29-0.62)	0.77(0.48-1.08)	0.012	0.003
Multivariable-adjusted 2 HR (95% CI)[5,2,4]	1.00 (ref.)	0.93 (0.71-1.23)	0.57 (0.39-0.84)	0.97 (0.64-1.47)	0.325	0.005
Myocardial infarction						
No. of cases	55	25	15	7		
Multivariable-adjusted 1 HR (95% CI)[1,2,4]	1.00 (ref.)	0.97(0.74-1.28)	0.65(0.47-0.90)	0.31(0.20-0.48)	<0.001	0.005
Multivariable-adjusted 2 HR (95% CI)[5,2,4]	1.00 (ref.)	1.09(0.83-1.44)	0.74(0.53-0.99)	0.35(0.22-0.54)	0.002	0.002
Cardiovascular death						
No. of cases	51	9	8	11		
Multivariable-adjusted 1 HR (95% CI)[1,2,4]	1.00 (ref.)	0.39(0.25-0.59)	0.49(0.31-0.75)	0.63(0.40-0.99)	0.355	<0.001
Multivariable-adjusted 2 HR (95% CI)[5,2,4]	1.00 (ref.)	0.42(0.27-0.65)	0.55(0.36-0.86)	0.75(0.46-1.19)	0.469	<0.001
Young Red Wine, mean (g/d)	0	1.69	9.03	24.03		
Primary end point						
No. of cases	188	36	23	25		
No. of person-years	19492	5080	3524	2690		
Unadjusted HR (95% CI)	1.00 (ref.)	0.82(0.70-0.95)	0.75(0.62-0.91)	1.03(0.83-1.28)	0.876	0.001
Multivariable-adjusted 1 HR (95% CI)[1,2,4]	1.00 (ref.)	0.78(0.66-0.92)	0.52(0.42-0.65)	0.55(0.43-0.71)	0.001	0.014
Multivariable-adjusted 2 HR (95% CI)[5,2,4]	1.00 (ref.)	0.89(0.75-1.05)	0.63(0.50-0.79)	0.66(0.51-0.87)	0.024	0.054
Ischemic stroke						
No. of cases	69	16	10	13		
Multivariable-adjusted 1 HR (95% CI)[1,2,4]	1.00 (ref.)	0.8(0.61-1.04)	0.45(0.30-0.66)	0.93(0.62-1.39)	0.238	<0.001
Multivariable-adjusted 2 HR (95% CI)[5,2,4]	1.00 (ref.)	0.98 (0.74-1.29)	0.60 (0.40-0.90)	1.21 (0.80-1.83)	0.865	0.001
Myocardial infarction						
No. of cases	71	14	10	7		
Multivariable-adjusted 1 HR (95% CI)[1,2,4]	1.00 (ref.)	0.90(0.70-1.17)	0.55(0.39-0.77)	0.29(0.18-0.46)	<0.001	0.066
Multivariable-adjusted 2 HR (95% CI)[5,2,4]	1.00 (ref.)	1.04(0.80-1.36)	0.64(0.45-0.90)	0.32(0.20-0.52)	0.001	0.029
Cardiovascular death						
No. of cases	60	6	6	7		
Multivariable-adjusted 1 HR (95% CI)[1,2,4]	1.00 (ref.)	0.41(0.27-0.61)	0.44(0.28-0.68)	0.57(0.35-0.91)	0.158	<0.001
Multivariable-adjusted 2 HR (95% CI)[5,2,4]	1.00 (ref.)	0.44(0.29-0.66)	0.50(0.32-0.80)	0.66(0.40-0.99)	0.267	<0.001
Old Red Wine, mean (g/d)	0	1.22	8.95	23.62		
Primary end point						
No. of cases	239	19	10	4		
No. of person-years	27075	2459	847	403		
Unadjusted HR (95% CI)	1.00 (ref.)	0.64(0.54-0.76)	0.83(0.57-1.21)	1.66(0.98-2.82)	0.025	0.001
Multivariable-adjusted 1 HR (95% CI)[1,2,4]	1.00 (ref.)	0.57(0.47-0.69)	0.55(0.35-0.86)	1.20(0.35-2.08)	0.128	0.001
Multivariable-adjusted 2 HR (95% CI)[5,2,4]	1.00 (ref.)	0.64(0.52-0.79)	0.62(0.40-0.97)	1.21(0.69-2.10)	0.13	0.03
Ischemic stroke						
No. of cases	100	4	3	1		
Multivariable-adjusted 1 HR (95% CI)[1,2,4]	1.00 (ref.)	0.32(0.22-0.47)	0.23(0.08-0.62)	0.36(0.08-1.52)	0.307	0.069
Multivariable-adjusted 2 HR (95% CI)[5,2,4]	1.00 (ref.)	0.33(0.21-0.51)	0.28(0.10-0.79)	0.45(0.10-1.93)	0.607	0.076
Myocardial infarction						
No. of cases	85	12	5	0		
Multivariable-adjusted 1 HR (95% CI)[1,2,4]	1.00 (ref.)	0.99(0.76-1.28)	0.71(0.36-1.40)	-	0.379	0.573
Multivariable-adjusted 2 HR (95% CI)[5,2,4]	1.00 (ref.)	1.22(0.92-1.61)	0.79(0.40-1.58)	-	0.34	0.332
Cardiovascular death						
No. of cases	69	4	2	4		
Multivariable-adjusted 1 HR (95% CI)[1,2,4]	1.00 (ref.)	0.24(0.13-0.44)	0.85(0.42-1.74)	0.9(0.05-2.95)	<0.001	<0.001
Multivariable-adjusted 2 HR (95% CI)[5,2,4]	1.00 (ref.)	0.27(0.14-0.50)	0.92(0.44-1.91)	1.05(0.05-2.82)	<0.001	<0.001
White Wine, mean (g/d)	0	0.80	8.82	22.49		
Primary end point						
No. of cases	240	27	3	2		
No. of person-years	27961	2419	265	141		
Unadjusted HR (95% CI)	1.00 (ref.)	0.82(0.67-0.99)	0.97(0.50-1.87)	0.86(0.27-2.68)	0.788	0.976

Multivariable-adjusted 1 HR (95% CI)[1,2,4]	1.00 (ref.)	0.80(0.64-0.99)	0.45(0.20-1.01)	0.49(0.12-2.00)	0.247	0.736
Multivariable-adjusted 2 HR (95% CI)[5,2,4]	1.00 (ref.)	0.85(0.68-1.07)	0.43(0.18-0.97)	0.50(0.12-2.02)	0.19	0.74
Ischemic stroke						
No. of cases	98	8	1	1		
Multivariable-adjusted 1 HR (95% CI)[1,2,4]	1.00 (ref.)	0.70(0.48-1.02)	0.84(0.26-2.66)	0.69(0.09-5.03)	0.705	0.711
Multivariable-adjusted 2 HR (95% CI)[5,2,4]	1.00 (ref.)	0.72 (0.48-1.09)	0.92 (0.29-2.92)	1.19 (0.16-8.74)	0.876	0.602
Myocardial infarction						
No. of cases	89	12	1	0		
Multivariable-adjusted 1 HR (95% CI)[1,2,4]	1.00 (ref.)	0.82(0.59-1.13)	0.19(0.02-1.41)	-	0.904	0.926
Multivariable-adjusted 2 HR (95% CI)[5,2,4]	1.00 (ref.)	0.97(0.69-1.35))	0.21(0.02-1.53)	-	0.909	0.928
Cardiovascular death						
No. of cases	68	9	1	1		
Multivariable-adjusted 1 HR (95% CI)[1,2,4]	1.00 (ref.)	0.84(0.53-1.33)	0.40(0.09-1.65)	1.28(0.17-9.56)	0.76	0.616
Multivariable-adjusted 2 HR (95% CI)[5,2,4]	1.00 (ref.)	0.87(0.54-1.39)	0.37(0.08-1.54)	1.14(0.15-8.55)	0.713	0.574
Beer, mean (g/d)	0	1.23	8.03	26.7		
Primary end point						
No. of cases	178	51	32	11		
No. of person-years	20694	6217	3478	398		
Unadjusted HR (95% CI)	1.00 (ref.)	0.79(0.69-0.90)	0.90(0.70-1.16)	1.94(1.34-2.82)	<0.001	<0.001
Multivariable-adjusted 1 HR (95% CI)[1,3,4]	1.00 (ref.)	0.67 (0.57-0.79)	0.70 (0.52-0.96)	1.58 (1.03-2.44)	0.022	<0.001
Multivariable-adjusted 2 HR (95% CI)[5,3,4]	1.00 (ref.)	0.75(0.62-0.88)	0.73(0.54-0.99)	1.71(1.11-2.64)	0.048	<0.001
Ischemic stroke						
No. of cases	76	15	13	4		
Multivariable-adjusted 1 HR (95% CI)[1,3,4]	1.00 (ref.)	0.55(0.42-0.72)	0.65(0.38-1.08)	1.94 (1.01-3.73)	0.081	<0.001
Multivariable-adjusted 2 HR (95% CI)[5,3,4]	1.00 (ref.)	0.68 (0.52-0.90)	0.78 (0.47-1.32)	2.26 (1.16-4.41)	0.054	0.001
Myocardial infarction						
No. of cases	62	24	11	5		
Multivariable-adjusted 1 HR (95% CI)[1,3,4]	1.00 (ref.)	0.73(0.57-0.94)	0.60(0.37-0.97)	1.28(0.63-2.60)	0.711	0.025
Multivariable-adjusted 2 HR (95% CI)[5,3,4]	1.00 (ref.)	0.81(0.63-1.05)	0.56(0.34-0.92)	1.26(0.61-2.58)	0.860	0.027
Cardiovascular death						
No. of cases	55	13	7	4		
Multivariable-adjusted 1 HR (95% CI)[1,3,4]	1.00 (ref.)	0.47(0.33-0.68)	0.91(0.53-1.58)	1.96(0.90-4.30)	0.016	<0.001
Multivariable-adjusted 2 HR (95% CI)[5,3,4]	1.00 (ref.)	0.47(0.32-0.68)	0.79(0.45-1.38)	2.11(0.95-4.65)	0.04	<0.001
Spirits, mean (g/d)	0	0.99	7.62	22.67		
Primary end point						
No. of cases	226	33	13	0		
No. of person-years	25635	3806	1277	68		
Unadjusted HR (95% CI)	1.00 (ref.)	1.10(0.94-1.28)	1.28 (0.88-1.87)	-	0.747	0.816
Multivariable-adjusted 1 HR (95% CI)[1,2,3]	1.00 (ref.)	0.87 (0.72-1.05)	1.02 (0.67-1.56)	-	0.885	0.849
Multivariable-adjusted 2 HR (95% CI)[5,2,3]	1.00 (ref.)	0.97 (0.80-1.18)	1.01 (0.66-1.54)	-	0.92	0.99
Ischemic Stroke						
No. of cases	91	11	6	0		
Multivariable-adjusted 1 HR (95% CI)[1,2,3]	1.00 (ref.)	1.01(0.77-1.44)	1.29(0.66-2.54)	-	0.910	0.907
Multivariable-adjusted 2 HR (95% CI)[5,2,3]	1.00 (ref.)	1.27 (0.92-1.75)	1.47 (0.74-2.92)	-	0.908	0.903
Myocardial infarction						
No. of cases	81	14	7	0		
Multivariable-adjusted 1 HR (95% CI)[1,2,3]	1.00 (ref.)	0.73(0.55-0.98)	1.12(0.60-2.02)	-	0.198	0.316
Multivariable-adjusted 2 HR (95% CI)[5,2,3]	1.00 (ref.)	0.80 (0.59-1.07)	0.97 (0.52-1.82)	-	0.211	0.248
Cardiovascular death						
No. of cases	66	8	5	0		
Multivariable-adjusted 1 HR (95% CI)[1,2,3]	1.00 (ref.)	0.73(0.49-1.10)	1.35(0.69-2.63)	-	0.005	0.008
Multivariable-adjusted 2 HR (95% CI)[5,2,3]	1.00 (ref.)	0.76(0.50-1.14)	1.12 (0.56-2.23)	-	0.023	0.063

HR: Hazard ratio; CI: Confidence interval.
[1]The multivariable HR was adjusted by age (continuous variable), physical activity, total energy intake, baseline body mass index, baseline waist circumference, smoking status (never, current or former smokers categorized in three groups: no smoking since 0 to 1 year, 1 to 5 years, more than 5 years), intake of [2]beer, [3]wine or [4]liquors and stratified according to recruitment center, sex, and intervention group.
[5]This model has been additionally adjusted by hypertension at baseline, dyslipidemia at baseline, diabetes at baseline, use of aspirin, antihypertensive drug, hypolipidemic drugs, oral hypoglycaemic agents, insulin, and vitamins intake; education (as two categories: primary school and high school and university studies); intake of protein, saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, cholesterol, [2]beer, [3]wine or [4]liquors (all as continuous variables).

Table 4. Stratified Analysis. Hazard Ratios (95% confidence intervals) of all-cause mortality within subgroups.

	Total Alcohol				
Groups according to alcohol intake (g/d)	0	>0-5	>5-15	>15-70	*p* for interaction
Sex					0.01
Men	1.00 (ref.)	0.37(0.26-0.51)	0.41(0.30-0.56)	0.49(0.36-0.66)	
Women	1.00 (ref.)	1.17(0.88-1.57)	1.02(0.70-1.48)	1.02(0.52-2.01)	
Age					0.50
<70 y	1.00 (ref.)	0.47(0.32-0.69)	0.45(0.30-0.69)	0.44(0.29-0.66)	
≥70 y	1.00 (ref.)	0.79(0.61-1.04)	0.77(0.57-1.04)	0.96(0.69-1.35)	
Diabetes					0.04
No	1.00 (ref.)	0.68(0.45-1.03)	0.76(0.50-1.16)	0.93(0.50-1.16)	
Yes	1.00 (ref.)	0.71(0.54-0.92)	0.65(0.48-0.87)	0.72(0.52-0.99)	
Hypercholesterolemia					0.54
No	1.00 (ref.)	0.59(0.42-0.84)	0.67(0.46-0.98)	0.62(0.42-0.93)	
Yes	1.00 (ref.)	0.80(0.59-1.07)	0.65(0.47-0.90)	0.90(0.64-1.26)	
Smoking					0.001
Never	1.00 (ref.)	1.08(0.81-1.44)	1.11(0.78-1.57)	1.32(0.80-2.18)	
Ever	1.00 (ref.)	0.40(0.29-0.57)	0.40(0.29-0.55)	0.49(0.36-0.66)	
Family history of early CHD					0.62
No	1.00 (ref.)	0.70(0.55-0.90)	0.68(0.52-0.89)	0.80(0.60-1.06)	
Yes	1.00 (ref.)	0.41(0.24-0.71)	0.27(0.13-0.56)	0.55(0.26-1.13)	
Education					0.57
Primary school	1.00 (ref.)	0.81(0.64-1.03)	0.69(0.52-0.91)	0.81(0.59-1.09)	
High school and university studies	1.00 (ref.)	0.39(0.23-0.66)	0.44(0.26-0.75)	0.46(0.27-0.78)	
Intervention group					0.001
Mediterranean Diet	1.00 (ref.)	0.66(0.52-0.84)	0.57(0.44-0.75)	0.59(0.44-0.79)	
Control	1.00 (ref.)	1.16(0.72-1.86)	1.33(0.80-2.20)	1.96(1.16-3.32)	

All adjusted by age (continuous variable), physical activity, total energy intake, baseline body mass index, baseline waist circumference, smoking status (never, current or former smokers categorized in three groups: no smoking since 0 to 1 year, 1 to 5 years, more than 5 years), hypertension at baseline, dyslipidemia at baseline, diabetes at baseline, use of aspirin, antihypertensive drug, hypolipidemic drugs, oral hypoglycaemic agents, insulin, and vitamins intake; education (as two categories: primary school and high school and university studies); intake of protein, saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, cholesterol, [1]beer , [2]wine, or [3]liquors (all as continuous variables).

Table 5. Stratified analysis of mortality. Hazard ratios of mortality (95% confidence intervals) according to the type of death and daily alcohol intake and dietary intervention

Groups according to alcohol intake (g/d)	n, cases	**Total Alcohol** 0	>0-5	>5-15	>15-70	*p* for interaction
Cardiovascular death						0.90
Mediterranean Diet	4786,53	1.00 (ref.)	0.94(0.57-1.55)	0.88(0.51-1.49)	0.87(0.51-1.49)	
Control	2368, 26	1.00 (ref.)	0.26(0.08-0.85)	0.85(0.31-2.31)	1.56(0.56-4.31)	
Cancer death						0.05
Mediterranean Diet	4786 ,93	1.00 (ref.)	0.83(0.55-1.24)	0.75(0.47-1.17)	0.77(0.48-1.23)	
Control	2368, 34	1.00 (ref.)	2.45(1.07-5.63)	2.25(0.91-5.51)	3.32(1.32-8.34)	
Death from other causes						<0.01
Mediterranean Diet	4786 ,68	1.00 (ref.)	0.41(0.27-0.61)	0.3(0.19-0.48)	0.35(0.21-0.58)	
Control	2368,	1.00 (ref.)	1.56(0.73-3.34)	1.51 (0.65-3.48)	2.30(0.94-5.64)	

All adjusted by age (continuous variable), physical activity, total energy intake, baseline body mass index, baseline waist circumference, smoking status (never, current or former smokers categorized in three groups: no smoking since 0 to 1 year, 1 to 5 years, more than 5 years), hypertension at baseline, dyslipidemia at baseline, diabetes at baseline, use of aspirin, antihypertensive drug, hypolipidemic drugs, oral hypoglycaemic agents, insulin, and vitamins intake; education (as two categories: primary school and high school and university studies); intake of protein, saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, cholesterol, (all as continuous variables), stratified according to recruitment center, sex, and intervention group.

Table 6. Stratified Analysis. Hazard Ratios (95% confidence intervals) of major cardiovascular events within subgroups.

	Total Alcohol				
Groups according to alcohol intake (g/d)	0	>0-5	>5-15	>15-70	*p* for interaction
Sex					0.07
Men	1.00 (ref.)	0.99(0.73-1.33)	0.68(0.50-0.93)	0.52(0.37-0.72)	
Women	1.00 (ref.)	0.75(0.57-0.97)	1.25(0.93-1.68)	0.53(0.25-1.11)	
Age					0.63
<70 y	1.00 (ref.)	0.99(0.74-1.33)	0.83(0.59-1.15)	0.69(0.48-0.98)	
≥70 y	1.00 (ref.)	0.79(0.60-1.04)	0.91(0.68-1.22)	0.40(0.26-0.61)	
Diabetes					0.96
No	1.00 (ref.)	0.83(0.59-1.18)	0.89(0.62-1.27)	0.39(0.25-0.60)	
Yes	1.00 (ref.)	0.99(0.78-1.26)	0.89(0.66-1.14)	0.72(0.52-0.99)	
Hypercholesterolemia					0.9
No	1.00 (ref.)	0.99(0.73-1.33)	0.90(0.64-1.27)	0.68(0.46-0.99)	
Yes	1.00 (ref.)	0.82(0.63-1.07)	0.81(0.61-1.07)	0.41(0.28-0.59)	
Smoking					0.02
Never	1.00 (ref.)	0.84(0.65-1.09)	1.46(1.10-1.93)	0.51(0.31-0.82)	
Ever	1.00 (ref.)	0.79(0.58-1.06)	0.46(0.33-0.64)	0.44(0.31-061)	
Family history of early CHD					0.02
No	1.00 (ref.)	0.92(0.74-1.14)	0.78(0.60-0.98)	0.55(0.41-0.73)	
Yes	1.00 (ref.)	0.75(0.47-1.19)	1.26(0.77-2.07)	0.44(0.22-0.88)	
Education					0.21
Primary school	1.00 (ref.)	0.83(0.67-1.02)	0.79(0.62-1.01)	0.63(0.47-0.84)	
High school and university studies	1.00 (ref.)	1.33(0.75-2.39)	0.85(0.46-1.57)	0.41(0.21-0.87)	
Intervention group					0.01
Mediterranean Diet	1.00 (ref.)	1.00(0.79-1.26)	0.96(0.74-1.24)	0.51(0.37-0.7)	
Control	1.00 (ref.)	0.81(0.58-1.13)	0.69(0.47-1.03)	0.70(0.45-1.10)	

All adjusted by age (continuous variable), physical activity, total energy intake, baseline body mass index, baseline waist circumference, smoking status (never, current or former smokers categorized in three groups: no smoking since 0 to 1 year, 1 to 5 years, more than 5 years), hypertension at baseline, dyslipidemia at baseline, diabetes at baseline, use of aspirin, antihypertensive drug, hypolipidemic drugs, oral hypoglycaemic agents, insulin, and vitamins intake; education (as two categories: primary school and high school and university studies); intake of protein, saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, cholesterol, [1]beer , [2]wine, or [3]liquors (all as continuous variables), stratified according to recruitment center, sex, and intervention group.

SUPPLEMENTAL TABLES

eTable 1. Stratified Analysis according to categories of cumulative daily intake of wine and beer. Hazard Ratios (95% confidence intervals) of all-cause mortality within subgroups.

	Wine[1,3]					Beer[2,3]				
Groups according to alcohol intake (g/d)	0	>0-5	>5-15	>15-70	***p* for interaction**	0	>0-5	>5-15	>15-70	***p* for interaction**
Sex					0.03					0.04
Men	1.00 (ref.)	0.44(0.33-0.58)	0.48(0.36-0.63)	0.62(0.47-0.81)		1.00 (ref.)	0.44(0.35-0.55)	0.53(0.38-0.74)	1.04(0.62-1.73)	
Women	1.00 (ref.)	1.30(0.97-1.73)	1.06(0.71-1.58)	0.97(0.44-2.12)		1.00 (ref.)	0.07(0.53-1.06)	0.05(0.18-1.4)	-	
Age					0.61					<0.001
<70 y	1.00 (ref.)	0.46(0.32-0.66)	0.48(0.33-0.71)	0.45(0.30-0.67)		1.00 (ref.)	0.54(0.40-0.73)	0.56(0.36-0.86)	0.62(0.30-1.28)	
≥70 y	1.00 (ref.)	0.86(0.66-1.12)	0.78(0.58-1.04)	1.14(0.82-1.57)		1.00 (ref.)	0.49(0.38-0.62)	0.51(0.31-0.82)	1.21(0.31-0.82)	
Diabetes					0.04					0.03
No	1.00 (ref.)	0.76(0.52-1.09)	0.66(0.44-0.99)	1.06(0.72-1.57)		1.00 (ref.)	0.55(0.41-0.75)	0.69(0.44-1.09)	1.33(0.58-3.03)	
Yes	1.00 (ref.)	0.80(0.62-1.03)	0.75(0.56-0.98)	0.77(0.54-0.99)		1.00 (ref.)	0.51(0.40-0.65)	0.56(0.36-0.88)	0.97(0.50-1.89)	
Hypercholesterolemia					0.2					<0.001
No	1.00 (ref.)	0.56(0.40-0.79)	0.81(0.57-1.15)	0.71(0.49-1.02)		1.00 (ref.)	0.41(0.31-0.53)	0.21(0.11-0.40)	0.25(0.06-1.07)	
Yes	1.00 (ref.)	0.89(0.68-1.17)	0.60(0.43-0.83)	0.94(0.67-1.33)		1.00 (ref.)	0.89(0.68-1.17)	0.60(0.43-0.83)	0.94(0.67-1.33)	
Smoking					0.007					0.32
Never	1.00 (ref.)	1.19(0.89-1.58)	1.22(0.86-1.74)	1.31(0.75-2.28)		1.00 (ref.)	0.5(0.36-0.71)	1.36(0.78-2.40)	-	
Ever	1.00 (ref.)	0.43(0.32-0.58)	0.43(0.32-0.58)	0.59(0.45-0.79)		1.00 (ref.)	0.50(0.40-0.63)	0.43(0.30-0.63)	0.93(0.55-1.56)	
Family history of early CHD					0.9					0.74
No	1.00 (ref.)	0.78(0.62-0.99)	0.65(0.50-0.85)	0.87(0.66-1.14)		1.00 (ref.)	0.53(0.43-0.65)	0.58(0.42-0.82)	1.10(0.65-1.86)	
Yes	1.00 (ref.)	0.36(0.21-0.62)	0.45(0.23-0.89)	0.75(0.36-1.57)		1.00 (ref.)	0.41(0.25-0.68)	0.60(0.26-1.39)	1.83(0.19-17.2)	
Education					0.05					0.26
Primary school	1.00 (ref.)	0.88(0.70-1.11)	0.70(0.53-0.92)	0.75(0.53-0.92)		1.00 (ref.)	0.57(0.46-0.72)	0.61(0.41-0.92)	1.77(1.00-3.12)	
High school and university studies	1.00 (ref.)	0.38(0.23-0.64)	0.50(0.30-0.82)	0.62(0.38-1.00)		1.00 (ref.)	0.40(0.28-0.57)	0.46(0.28-0.76)	0.27(0.06-1.16)	
Intervention group					<0.001					0.04
Mediterranean Diet	1.00 (ref.)	0.63(0.50-0.80)	0.57(0.44-0.73)	0.52(0.39-0.70)		1.00 (ref.)	0.47(0.38-0.59)	0.45(0.31-0.66)	1.2(0.7-2.06)	
Control	1.00 (ref.)	1.60(1.02-2.48)	1.64(0.98-2.72)	3.31(2.01-5.45)		1.00 (ref.)	0.86(0.59-1.23)	1.05(0.62-1.80)	0.30(0.04-2.27)	

HR: Hazard ratio; CI: Confidence interval.[1]The multivariable HR was adjusted by age (continuous variable), physical activity, total energy intake, baseline body mass index, baseline waist circumference, smoking status (never, current or former smokers categorized in three groups: no smoking since 0 to 1 year, 1 to 5 years, more than 5 years), intake of [2]beer, [3]wine or [4]liquors and stratified according to recruitment center, sex, and intervention group.

[5]This model has been additionally adjusted by hypertension at baseline, dyslipidemia at baseline, diabetes at baseline, use of aspirin, antihypertensive drug, hypolipidemic drugs, oral hypoglycaemic agents, insulin, and vitamins intake; education (as two categories: primary school and high school and university studies); intake of protein, saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, cholesterol, [2]beer, [3]wine or [4]liquors (all as continuous variables).

All adjusted by age (continuous variable), physical activity, total energy intake, baseline body mass index, baseline waist circumference, smoking status (never, current or former smokers categorized in three groups: no smoking since 0 to 1 year, 1 to 5 years, more than 5 years), hypertension at baseline, dyslipidemia at baseline, diabetes at baseline, use of aspirin, antihypertensive drug, hypolipidemic drugs, oral hypoglycaemic agents, insulin, and vitamins intake; education (as two categories: primary school and high school and university studies); intake of protein, saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, cholesterol, [1]beer , [2]wine, or [3]liquors (all as continuous variables).

eTable 2. Stratified analysis of mortality according to categories of cumulative daily intake of wine and beer. Hazard ratios of mortality (95% confidence intervals) according to the type of death and daily alcohol intake and dietary intervention

	Wine					Beer				
Groups according to alcohol intake (g/d)	0	>0-5	>5-15	>15-70	***p* for interaction**	0	>0-5	>5-15	>15-70	*p* for interaction
Cardiovascular death					0.60					0.05
Mediterranean Diet	1.00 (ref.)	0.58(0.36-0.94)	0.70(0.44-1.13)	0.60(0.34-1.05)		1.00 (ref.)	0.39(0.26-0.61)	0.36(0.17-0.77)	1.59(0.66-3.78)	
Control	1.00 (ref.)	0.26(0.08-0.85)	0.86(0.31-2.32)	1.53(0.55-4.22)		1.00 (ref.)	0.79(0.57-1.08)	0.58(0.30-1.12)	1.43(0.50-4.09)	
Cancer death					0.13					0.33
Mediterranean Diet	1.00 (ref.)	0.97(0.67-1.4)	0.58(0.37-0.91)	0.70(0.44-0.99)		1.00 (ref.)	0.39(0.26-0.61)	0.36(0.17-0.77)	1.59(0.66-3.79)	
Control	1.00 (ref.)	3.76(1.69-8.38)	3.16(1.30-7.68)	6.0(2.49-14.4)		1.00 (ref.)	0.59(0.23-1.52)	2.55(0.90-7.17)	6.43(0.67-6.1)	
Death from other causes					<0.001					<0.001
Mediterranean Diet	1.00 (ref.)	0.47(0.31-0.73)	0.46(0.30-0.72)	0.50(0.27-0.86)		1.00 (ref.)	0.43(0.29-0.63)	0.42(0.20-0.86)	-	
Control	1.00 (ref.)	1.98(0.99-3.96)	1.95(0.87-4.38)	3.42(1.48-7.9)		1.00 (ref.)	0.74(0.42-1.32)	1.4(0.7-2.97)	-	

All adjusted by age (continuous variable), physical activity, total energy intake, baseline body mass index, baseline waist circumference, smoking status (never, current or former smokers categorized in three groups: no smoking since 0 to 1 year, 1 to 5 years, more than 5 years), hypertension at baseline, dyslipidemia at baseline, diabetes at baseline, use of aspirin, antihypertensive drug, hypolipidemic drugs, oral hypoglycaemic agents, insulin, and vitamins intake; education (as two categories: primary school and high school and university studies); intake of protein, saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, cholesterol, (all as continuous variables), stratified according to recruitment center, sex, and intervention group.

eTable 3. Stratified Analysis according to categories of cumulative daily intake of wine and beer. Hazard Ratios (95% confidence intervals) of major cardiovascular events within subgroups.

	Wine[1,3]					Beer[2,3]				
Groups according to alcohol intake (g/d)	0	>0-5	>5-15	>15-70	***p* for interaction**	0	>0-5	>5-15	>15-70	***p* for interaction**
Sex					0.19					0.28
Men	1.00 (ref.)	0.96(0.74-1.25)	0.56(0.42-0.75)	0.56(0.41-0.75)		1.00 (ref.)	0.60(0.49-0.75)	0.57(0.40-0.81)	1.59(1.01-1.05)	
Women	1.00 (ref.)	0.87(0.66-1.16)	1.49(1.08-2.05)	0.31(0.09-1.01)		1.00 (ref.)	0.09(0.72-1.29)	0.01(0.77-2.77)	-	
Age					0.31					0.05
<70 y	1.00 (ref.)	1.09(0.83-1.44)	0.62(0.44-0.88)	0.75(0.52-1.07)		1.00 (ref.)	0.84(0.67-1.08)	0.76(0.51-1.13)	1.81(1.09-3.01)	
≥70 y	1.00 (ref.)	0.89(0.68-1.17)	1.09(0.81-1.46)	0.48(0.30-0.74)		1.00 (ref.)	0.58(0.44-0.77)	0.67(0.39-1.12)	1.05(0.36-3.02)	
Diabetes					0.51					0.62
No	1.00 (ref.)	1.07(0.77-1.47)	0.79(0.55-1.15)	0.51(0.32-0.79)		1.00 (ref.)	0.51(0.37-0.69)	0.61(0.38-1.00)	1.30(0.64-2.60)	
Yes	1.00 (ref.)	0.99(0.78-1.26)	0.85(0.64-1.12)	0.75(0.64-1.12)		1.00 (ref.)	0.90(0.72-.12)	0.78(0.52-1.17)	1.62(0.86-3.07)	
Hypercholesterolemia					0.03					<0.01
No	1.00 (ref.)	0.90(0.67-1.20)	0.96(0.69-1.34)	0.78(0.53-1.14)		1.00 (ref.)	0.52(0.40-0.68)	0.45(0.27-0.75)	0.75(0.29-1.9)	
Yes	1.00 (ref.)	0.98(0.76-1.26)	0.62(0.45-0.84)	0.39(0.26-0.58)		1.00 (ref.)	0.99(0.78-1.26)	1.10(0.73-1.64)	2.45(1.41-4.25)	
Smoking					0.04					0.23
Never	1.00 (ref.)	0.91(0.69-1.19)	1.47(1.09-1.98)	0.47(0.27-0.81)		1.00 (ref.)	1.0(0.81-1.36)	0.6(0.29-1.3)	-	
Ever	1.00 (ref.)	0.87(0.66-1.14)	0.46(0.33-0.63)	0.54(0.39-0.75)		1.00 (ref.)	0.57(0.45-0.73)	0.72(0.51-1.02)	1.63(1.02-2.60)	
Family history of early CHD					0.04					0.13
No	1.00 (ref.)	0.95(0.77-1.17)	0.71(0.55-0.91)	0.59(0.44-0.79)		1.00 (ref.)	0.75(0.62-0.91)	0.75(0.53-1.04)	1.85(1.15-2.96)	
Yes	1.00 (ref.)	1.04(0.66-1.63)	1.25(0.73-2.13)	0.35(0.15-0.80)		1.00 (ref.)	0.67(0.41-1.08)	0.71(0.32-1.59)	1.66(0.32-8.62)	
Education					0.6					0.7
Primary school	1.00 (ref.)	0.92(0.74-1.13)	0.76(0.59-0.98)	0.63(0.46-0.85)		1.00 (ref.)	0.81(0.71-1.06)	0.90(0.63-1.27)	1.78(1.01-3.12)	
High school and university studies	1.00 (ref.)	1.76(1.05-2.94)	0.92(0.52-1.62)	0.75(0.40-1.38)		1.00 (ref.)	0.33(0.22-0051)	0.35(0.17-0.72)	1.56(0.68-3.57)	
Intervention group					0.01					0.03
Mediterranean Diet	1.00 (ref.)	1.07(0.86-1.35)	0.86(0.66-1.11)	0.53(0.38-0.73)		1.00 (ref.)	0.69(0.56-0.85)	0.77(0.54-1.09)	1.87(1.13-3.08)	
Control	1.00 (ref.)	0.88(0.62-0.88)	0.67(0.44-1.03)	0.79(0.49-1.28)		1.00 (ref.)	0.79(0.57-1.08)	0.58(0.30-1.12)	1.43(0.50-4.09)	

All adjusted by age (continuous variable), physical activity, total energy intake, baseline body mass index, baseline waist circumference, smoking status (never, current or former smokers categorized in three groups: no smoking since 0 to 1 year, 1 to 5 years, more than 5 years), hypertension at baseline, dyslipidemia at baseline, diabetes at baseline, use of aspirin, antihypertensive drug, hypolipidemic drugs, oral hypoglycaemic agents, insulin, and vitamins intake; education (as two categories: primary school and high school and university studies); intake of protein, saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, and cholesterol (all as continuous variables), stratified according to recruitment center and intervention group.

eTable 4. Cox proportional hazard ratios for all-cause mortality and cardiovascular events in men participants with preferential consumption of wine or beer. Participants consumed at least 75% of the total alcohol as wine or beer.

	Wine			
Groups according to alcohol intake (g/d)	0	>0-5	5-15	>15-70
Mortality				
No. of person-years	2045	1166	1623	2211
No. cases	47	16	24	38
Multivariable-adjusted 2 HR (95% CI)	1.00 (ref.)	0.43(0.28-0.65)	0.49(0.35-0.69)	0.61(0.43-0.87)
Primary End Point				
No. of person-years	2037	1162	1620	2215
No. cases	34	19	20	20
Multivariable-adjusted 2 HR (95% CI)	1.00 (ref.)	1.16(0.82-1.64)	0.69(0.48-0.98)	0.57(0.38-0.85)
Ischemic stroke				
Multivariable-adjusted 2 HR (95% CI)	1.00 (ref.)	1.08(0.57-2.05)	1.07(0.55-2.06)	1.03(0.49-2.14)
Myocardial Infarction				
Multivariable-adjusted 2 HR (95% CI)	1.00 (ref.)	1.43(0.89-2.3)	0.50(0.29-0.88)	0.24(0.12-0.46)
Cardiovascualr death				
Multivariable-adjusted 2 HR (95% CI)	1.00 (ref.)	0.47(0.19-1.11)	0.47(0.22-0.98)	0.90(0.44-1.86)

4.3.3. Publication 8. Effect of dietary polyphenols on cardiovascular risk.

Rosa Maria Lamuela Raventós, **Paola Quifer-Rada**. *Heart,* 2016;102(17):1340-1

This editorial letter for the journal Heart focuses on the evidences of dietary polyphenols in cardiovascular diseases prevention. It especially focuses in their possible implication on endothelium-dependent vasodilation mechanism related to blood pressure maintenance.

Hypertension is one of the main cardiovascular risk factors in the elderly population and its control has become a key health priority for public health organisations. The effect of dietary polyphenols on cardiovascular risk parameters has been clearly demonstrated in epidemiological and intervention nutritional trials with food naturally rich in polyphenols such as virgin olive oil, cocoa, berries, and wine. Based on this evidence, two health claims have been approved by the European Food Safety Authority about the effect of polyphenols on cardiovascular risk factors. Noad *et al*[107] demonstrate in a prospective nutritional intervention trial that a reasonable daily diet rich in polyphenol containing food (six portions of fruit and vegetables, including one portion of berries/day, and 50 g of dark chocolate) improves microvascular function in hypertensive participants, with a release of endothelium-dependent acetylcholine, an improved vasodilator response, and lower systolic blood pressure. In another experimental studies, dietary polyphenols have been shown to stimulate the secretion of endothelial NO, potentially decreasing blood pressure. Moreover, polyphenols have been shown to increase intracellular free calcium concentrations, which activates endothelial NO synthase.

Effect of dietary polyphenols on cardiovascular risk

Rosa M Lamuela-Raventos,[1,2] Paola Quifer-Rada[1,2]

Hypertension is one of the main cardiovascular risk factors in the elderly population and its control has become a key health priority for public health organisations. The adoption of a heart-healthy lifestyle is recommended, including a diet rich in fruits and vegetables, maintaining a body mass index between 18.5 and 24.9 kg/m^2, and performing physical exercise.

Research on polyphenol health effects began relatively late, not until 1995, probably because they are not essential nutrients and have very diverse chemical structures, with more than 800 different organic structures found in nature.[1] This hampers their analysis in foods and even more so in biological fluids, since they have low bioavailability. Another complication is that polyphenols can be metabolised by intestine or hepatic cells, or by intestinal microbiota when they arrive in the large intestine. Over the last two decades, the literature on polyphenols has grown exponentially following the recognition of their properties, with evidence for their potential beneficial effects on health being reported. The effect of dietary polyphenols on cardiovascular risk parameters has been clearly demonstrated in epidemiological and intervention nutritional trials with food naturally rich in polyphenols such as virgin olive oil, cocoa, berries, and wine. Based on this evidence, two health claims have been approved by the European Food Safety Authority about the effect of polyphenols on cardiovascular risk factors. The first, published 5 years ago, concerns the protective effect of hydroxytyrosol and its derivatives (oleuropein and secoiridois) from olives or virgin olive oils on low-density lipoprotein (LDL) particles against oxidative damage.[2] One year later, in 2012, another health claim was approved regarding the effect of cocoa flavanols on the maintenance of normal endothelium-dependent vasodilation.[3]

Noad *et al*[4] demonstrate in a prospective nutritional intervention trial that a reasonable daily diet rich in polyphenol-containing food (six portions of fruit and vegetables, including one portion of berries/day, and 50 g of dark chocolate) improves microvascular function in hypertensive participants, with a release of endothelium-dependent acetylcholine, an improved vasodilator response, and lower systolic blood pressure. This finding is in agreement with previous reports describing the vasodilator effects of phenolic compounds.[5 6] In experimental studies, dietary polyphenols have been shown to stimulate the secretion of endothelial nitric oxide (NO), potentially decreasing blood pressure. Further, numerous observational and short- or long-term intervention studies have demonstrated in various populations that the consumption of polyphenol-rich food is inversely correlated with blood pressure levels. However, most of them focus on a single source of polyphenols or food item. The impact of overall polyphenol-rich dietary patterns on microvascular function has been assessed in hypertensive subjects in a robustly designed trial by Noad *et al*.[4]

Acetylcholine (Ach) exerts a direct effect on vascular function by increasing NO production, which signals the surrounding smooth muscle to relax, leading to vasodilation. Although endothelium-derived NO is

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the most important mediator of ACh-induced relaxation of rat aortic ring preparations, vasorelaxation may also involve prostanoids.[7] Moreover, polyphenols have been shown to increase intracellular free calcium concentrations, which activates endothelial NO synthase. Although a causal relationship between ACh release and the observed decrease in blood pressure induced by polyphenols seems plausible, it was not demonstrated in the study by Noad *et al*. Polyphenols may exert multiple effects,[8] since they could bind to different metabolically active enzymes and/or receptors, so it should be clarified whether the decrease in blood pressure is causally related to the increase in Ach.

Nevertheless, other health effects of a polyphenol-rich diet have been observed by Noad *et al*, including a decrease in total cholesterol and lower LDL levels, although other food constituents may play a role, since fruit and vegetables are rich in fibre, vitamin C, and minerals (such as potassium and magnesium). Noad *et al* observed a significant increase in carotenes and vitamin C in plasma in the polyphenol-rich diet group, so these antioxidants or a possible synergism among them could also explain the beneficial effects observed. However, plausible mechanisms leading to increases in Ach and other positive changes remain to be elucidated.

Increased awareness of the benefits of healthy dietary habits by patients and their caregivers should foster their adoption. Lifestyle and dietary interventions minimise dependency on pharmacological antihypertensive therapies, thus avoiding the adverse effects and costs of drug use.

Contributors RML-R and PQ-R have both participated in the writing process of this editorial.

Competing interests None declared.

Provenance and peer review Commissioned; internally peer reviewed.

To cite Lamuela-Raventos RM, Quifer-Rada P. *Heart* 2016;**102**:1340–1341.

Published Online First 20 June 2016

▶ http://dx.doi.org/10.1136/heartjnl-2015-309218

Heart 2016;**102**:1340–1341.
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5. GLOBAL DISCUSSION

Beer is by far the most widely consumed alcoholic beverage in the world and it has been part of the human diet since at least 5000 BC. Moderate alcohol consumption in general has been inversely associated with incident cardiovascular disease in observational studies. However, it has been suggested that fermented beverages such as red wine and beer may confer greater protective effects than spirits owing to their higher polyphenolic content. It has been shown that daily intake of polyphenols may reduce the incidence of chronic diseases such as cancer, type-II diabetes, cardiovascular diseases and neurodegenerative diseases[4,5]. Beer phenolic profile is very diverse since about 70-80% of the polyphenols come from malt, and the remaining 30-20% come from hops[6]. And the main beer phenolic compounds are hydroxybenzoic acids, cinnamic acids such as ferulic acid, and flavonols[108]. In this context, the hypothesis raised in this thesis is that moderate beer consumption may reduce cardiovascular disease risk. In order to evaluate the truthfulness of the hypothesis, a biomarker of beer consumption was needed to measure compliance of volunteers in clinical studies more objectively. Thus, one of the main objectives of this thesis was to evaluate prenylflavonoids as biomarkers of beer consumption and asses its effectivity. Moreover, the cardioprotective effect of moderate beer intake was assessed in two studies: one randomized, crossover feeding trial and one observational cohort using the randomized controlled PREDIMED trial data.

In the first work presented in this thesis, we identified the full range of polyphenols found in beer by high-resolution mass spectrometry in order to comprehend greater the phenolic diversity in beer. In this work, 47 phenolic compounds were identified using high mass accuracy and confirmed by product ion scan experiments. Polyphenols identified include simple phenolic acids, hydroxycinnamoylquinic acids, flavanols, flavonols, flavones, alkylmethoxyphenols, alpha- and iso-alpha-acids, hydroxyphenylacetic acids and prenylflavanoids. As far as we know, 7 of these had never been described before in beer: feruloylquinic acid, caffeic acid-*O*-hexoside, coumaric acid-*O*-hexoside, sinapic acid-*O*-hexoside, catechin-*O*-dihexoside, kaempferol-*O*-hexoside, and apigenin-*C*-hexoside-pentoside. However, other polyphenols reported in other works, such as proanthocyanidins[108–110], could not be found possibly due to the chromatographic conditions.

In this thesis, we proposed hops prenylflavonoids as biomarkers of beer consumption due to its specificity since they are mainly consumed as beer in a habitual diet. Female inflorescences of hops used in brewing are particularly rich in XN. However, during the brewing process, XN isomerizes into IX, resulting in beer having larger amounts of IX than XN[57]. IX is further converted

into 8-prenylnaringenin (8PN) in the distal colon by an *O*-demethylation catalyzed by gut microbiota[111,112].

One of the conditions of nutritional biomarkers to be considered a good biomarker is to have a robust quantification method[83,84], however most of the methods to quantify prenylflavonoids reported previously failed to account the instability of prenylflavonoids in acidic medium[111,113–117]. Thus, we developed a robust method to quantify prenylflavonoids in urine by LC-MS/MS and it was optimized to maintain analyte stability throughout the analytical process (Publication 2). This new method differs from the others because it considers all variables that could affect analytes quantification and stability such as chromatographic mobile phases pH, pH during extraction, and the use of an antioxidant. The method was fully validated following the guidance for industry of the US Food and Drug Administration for the Validation of Bioanalytical Methods[118]. After evaluating the pH effect on reproducibility, accuracy, precision and stability in the analysis of IX, XN and 8N, it was concluded that a reconstitution medium and mobile phase at pH 7.0 is required for the analysis of prenylflavanoids to minimize analyte degradation. In contrast, all methods previously published had used acidic mobile phases and reconstitution media after SPE[111,113–117]. Thus, the new proposed method proved to be selective, accurate and precise with great recovery rates (>97%). Low matrix effect were obtained due to the use of a mixed-mode cation-exchange SPE capable to remove interferences from urine more thoroughly without compromising analyte recovery. Consequently, the limits of detection (LOD) and limits of quantification (LOQ) obtained were much lower than those of previously published methods[113,114]. Addition of ascorbic acid as antioxidant helped to maintain analytes stabile for at least 24 hours. Moreover, the new method allowed identifying and quantifying IX, XN and 8PN in human urine from volunteers who drank a moderate dose (330 mL) of beer. IX showed to be the only beer prenylflavonoid able to discriminate between beer and non-beer consumers. Polyphenols present in biological samples are mainly conjugated (glucuronidated, sulfated or methylated) then, prenylflavonoids of beer in urine samples are expected to be conjugated. The quantification of conjugated polyphenol metabolites in biological samples is hampered by the unavailability of standards. The most common analytical technique used is enzymatic hydrolysis, which breaks the *O*-glucuronide or *O*-sulfate bond and quantifies the polyphenol precursor. Since enzymatic hydrolysis conditions may be harmful for prenylflavonoids stability, we assessed their possible degradation during this process (Publication 3). We observed that both temperature (37 ºC) and pH (5.0) regularly used in enzymatic hydrolysis decreased prenylflavonoids recovery by 23%, 50% and 37% for 8PN, IX and XN, respectively. Moreover, higher amounts of enzyme used lead to a lower recovery of prenylflavonoids. Thus, enzymatic

hydrolysis may not be a good analytical technique to quantify conjugated prenylflavonoids in biological samples.

New biomarkers need to be validated in two stages: (i) in dose-response controlled clinical trials to identify the range of intake in which the biomarker is reliable, and (ii) in free-living populations to evaluate the suitability of the biomarker in a habitual diet[73]. Accordingly, the effectivity of IX as biomarker of beer consumption was tested in three studies: a dose-response clinical trial including young volunteers who drank three doses of beer, an intervention clinical trial with participants at high cardiovascular risk who received 30 g of ethanol/day as gin or beer, or an equivalent amount of polyphenols in non-alcoholic beer for 4 weeks and a cohort study of a free-living population (PREDIMED) (Publication 4). Unlike other polyphenol-based biomarkers[81,119,120], IX showed to be specific to only one dietary component (beer), which makes it as an ideal and highly sensitive biomarker of beer intake. In fact, a cutoff point of 0.48 µg IX/g creatinine in the ROC curve model allowed a perfect discrimination between beer and non-beer drinkers with a sensitivity of 98% and specificity of 96%; positive predictive value of 99%, and negative predictive value of 96%. This cutoff point is extremely low compared to other polyphenol-based biomarkers. For example, resveratrol metabolites were proposed to be biomarkers of wine consumption, the cutoff point that provided optimized sensitivity and specificity for the identification of wine consumers was 33 µg/g creatinine using data from a controlled clinical trial[119] and 151 µg/g creatinine using data from a free-living subjects study (PREDIMED)[121]. The area under the curve (AUC) of the ROC model using data from the PREDIMED study was 0.904 (95% CI, 0.797-1.00) with a sensitivity of 67%, and specificity of 100% which means that IX is a highly accurate biomarker of beer intake. IX also proved to be a good biomarker of non-alcoholic beer consumption. IX urinary excretion increased linearly with the dose size in male volunteers, however a saturation behavior was observed after a single dose of 1 beer (330 mL) in some women.

In order to discovery other novel biomarkers of beer consumption, we embarked an untargeted analysis using LC-HRMS Orbitrap in urine samples from an open, randomized, crossover, controlled trial with three nutritional interventions consisting of beer, non-alcoholic beer and gin. This discovery-driven approach was also used to study the urinary metabolomics changes related to the beverage type consumed (Publication 5). We observed that daily beer and non-alcoholic beer consumption changed the urinary metabolome according to the PLS-DA models, whereas changes after gin consumption were not significant. Three hop alpha acids identified had potential use as biomarkers of beer consumption, nevertheless humilinone was found to be the more suitable. Humulinone is an oxidized product of humulone normally found in beer[122] however, to confirm its effectivity as biomarker of beer consumption, a validation, as performed

for isoxanthohumol, is required. The untargeted approach also revealed that hydroxyadipic acid excretion increased around 3- and 2.5-fold after the beer and non-alcoholic beer intervention, respectively. Hydroxyadipic acid is a dicarboxylic acid formed by fatty acid ω-oxidation. Beer polyphenols and isohumulones may activate PPARα and increase liver fatty acid β- and ω-oxidation, resulting in an enhanced excretion of urinary hydroxyapidic acid. It has been shown that other polyphenols like catechin, genistein and citrus polymethoxylated flavones we able to activate PPARα[123–125]. Urinary excretion of 4-guanidinobutanoic acid also increased during beer and non-alcoholic beer interventions. 4-Guanidinobutanoic acid is a common urinary metabolite arising from the metabolism of arginine and proline (creatinine pathway) and is also related to nitric oxide production which is involved in the blood pressure regulation.

Publication 6 and 7 are focused on studying moderate beer consumption and cardiovascular disease risk. The publication 6 aimed to evaluate the effects of ethanol and the phenolic compounds of beer on classical and novel cardiovascular risk factors in participants at high cardiovascular risk. Moderate alcohol consumption, as beer or gin, improved cholesterol profile by increasing serum HDL-cholesterol, ApoA-I and ApoA-II. Moreover, it increased adiponectin levels, which is involved in glucose regulation and fatty acid oxidation, and decreased serum fibrinogen. Moderate beer intake showed greater cardioprotective effects than gin (an alcoholic polyphenol free beverage) by increasing serum concentrations of IL-1ra, decreased IL-5, and decreased lymphocyte expression of LFA-1 and SLe^x and monocyte expression of SLe^x and CCR2. However, when we compared these results with those observed after moderate wine intake[126,127], the protective effects of moderate wine intake (especially red wine) were higher than those observed after beer intake, suggesting that the non-alcoholic fraction of beer may be less cardioprotective than that of wine. On the other hand, systolic blood pressure, homocysteine and several biomarkers of inflammation decreased only after the non-alcoholic beer intervention, and these effects should be attributed to the non-alcoholic fraction of the beer, mainly polyphenols. Thus, no synergistic effects were observed between the alcoholic and the non-alcoholic fraction of beer in any of the outcomes studied.

The PREDIMED study is a large, multicenter, randomized, parallel group and controlled trial aimed at assessing the effects of two Mediterranean diets, supplemented with either extra virgin olive oil or mixed nuts, versus a low-fat control diet on cardiovascular outcomes in individuals at high cardiovascular risk. We carried out a prospective study aimed at assessing differential associations of wine, beer and spirit consumption with all-cause mortality and incidence of cardiovascular events within the context of the PREDIMED trial. In this pre-specified analysis, 7154 asymptomatic subjects at high cardiovascular risk consuming low and moderate amounts of wine and beer had a 38% reduction in the risk of all-cause mortality after a mean

follow-up of 4.8 years. Wine drinking at moderate and high levels was associated with a 71% reduction of cardiovascular disease events, while beer at low and moderate intake reduced cardiovascular events by 27%, but increased risk by 71% at high doses (>15 g of alcohol/d). Importantly, the effects of alcohol intake (either wine or beer) varied depending on the dietary pattern. Alcohol intake at >15 g/d in subjects assigned to the Mediterranean diet intervention was associated with a 41% reduced risk of all-cause mortality. By contrast, alcohol intake by those allocated to the low-fat diet intervention had no significant effects on all cause-mortality at levels of >0-5 and >5-15 g/d, but increased risk by 96% at higher levels (>15 g /day). The increased mortality risk in non-drinkers observed in our study is consistent with the J-shaped dose-response relationship widely reported in observational studies[63,128,129]. Some systematic reviews have concluded that beverage type is relatively unimportant in the association of alcohol and mortality and cardiovascular events. However, two other recent meta-analyses that evaluated different alcoholic beverages reached a different conclusion. One indicated that low-to-moderate wine and beer consumption was indeed associated with a decrease in cardiovascular events and mortality[130], and the other concluded that consumption of wine, but not beer or spirits, was inversely associated with cardiovascular risk[62]. The results of the current study were in the line with those of these last meta-analyses. In conclusion, we have observed that a moderate consumption of alcoholic beverages (wine and beer) is inversely associated with total mortality and the risk of cardiovascular events. However, the protective effects of wine seems to be higher than those of beer, and, more interestingly, these effects varied depending on the dietary pattern followed by the participants.

6. CONCLUSIONS

- ✓ 47 phenolic compounds were identified in beer by high-resolution mass spectrometry including simple phenolic acids, hydroxycinnamoylquinics, flavanols, flavonols, flavones, alkylmethoxyphenols, alpha- and iso-alpha-acids, hydroxyphenylacetic acids and prenylflavonoids.
- ✓ Feruloylquinic acid, caffeic acid-*O*-hexoside, coumaric acid-*O*-hexoside, sinapic acid-*O*-hexoside, catechin-*O*-dihexoside, kaempferol-*O*-hexoside, and apigenin-*C*-hexoside-pentoside were identified in beer for the first time.
- ✓ A new robust and specific method to quantify prenylflavonoids from beer in urine samples by LC-MS/MS was developed and fully validated.
- ✓ Prenylflavonoids from beer recovery decrease during enzymatic hydrolysis due to the procedure conditions such as temperature and pH.
- ✓ Isoxanthohumol probe to be a specific and accurate biomarker of beer consumption.
- ✓ 8-prenylnaringenin and xanthohumol are not good biomarkers of beer consumption due to the low concentration found in urine.
- ✓ Daily beer and non-alcoholic beer consumption changed the urinary metabolome, whereas changes after gin consumption were not significant.
- ✓ The non-alcoholic fraction of beer may increase hydroxyadipic excretion which might indicate that beer polyphenols and isohumulones activate PPARα and increase liver fatty acid β- and ω-oxidation.
- ✓ Urinary excretion of 4-guanidinobutanoic acid increased after a regular and daily intake of beer and non-alcoholic beer.
- ✓ The discovery-driven approach revealed that humulinone is a potential new biomarker of beer consumption.
- ✓ Moderate alcohol consumption (as gin or beer) increased serum HDL-cholesterol, ApoA-I, ApoA-II and adiponectin, and decreased serum fibrinogen.
- ✓ The non-alcoholic fraction of beer (mainly polyphenols) reduces leukocyte adhesion molecules and inflammatory biomarkers by increasing serum concentrations of IL-1ra, decreasing IL-5, decreasing lymphocyte expression of LFA-1 and SLe^x and monocyte expression of SLe^x and CCR2 and decreasing TNFβ and IL-15 plasma concentrations.
- ✓ Systolic blood pressure, homocysteine and several biomarkers of inflammation decreased only after the non-alcoholic beer intervention, and these effects should be attributed to the non-alcoholic fraction of the beer.

- ✓ No synergistic effects were observed between the alcoholic and the non-alcoholic fraction of beer in any of the outcomes studied.
- ✓ Light (< 5 g alcohol/day) and moderate (5-15 g alcohol/day) drinking of total wine, red wine, and beer was inversely associated with all-cause mortality, with HRs of 0.70 (0.56-0.88), 0.62 (0.49-0.78), and 0.62 (0.67-0.84), respectively, compared to non-drinkers.
- ✓ Light and moderate beer consumption was associated with a lower incidence of cardiovascular events [HR: 0.75 (0.62-0.88)].

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127. Chiva-Blanch, G. *et al.* Differential effects of polyphenols and alcohol of red wine on the expression of adhesion molecules and inflammatory cytokines related to atherosclerosis: a randomized clinical trial. *Am. J. Clin. Nutr.* **95,** 326–334 (2012).
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8. ANNEX

8.1 Other research articles

Publication 9

Authors: Heras, R.M.L.; Quifer-Rada, P.; Andrés, A.; Lamuela-Raventós, R.

Title: Polyphenolic profile of persimmon leaves by high resolution mass spectrometry (LC-ESI-LTQ-Orbitrap-MS)

Journal: Journal of Functional Foods

Volume: 23 **Number:** --- **Pages, Initial:** 370 **final:** 377 **Year:** 2016

Publication 10

Authors: Orrego-Lagarón, N.; Martínez-Huélamo, M.; Quifer-Rada, P.; Lamuela-Raventós, RM.; Escribano-Ferrer, E.

Title: Absorption and disposition of naringenin and quercetin after simultaneous administration via intestinal perfusion in mice

Journal: Food and Function

Volume: 7 **Number:** 9 **Pages, Initial:** 3880 **final:** 3889 **Year:** 2016

Publication 11

Authors: Quifer-Rada, P.; Choy, Y.Y.; Calvert, C.C.; Waterhouse, A.L.; Lamuela-Raventos, R.M.

Title: Use of metabolomics and lipidomics to evaluate the hypocholestreolemic effect of Proanthocyanidins from grape seed in a pig model.

Journal: Molecular Nutrition & Food Research

Volume: 60 **Number:** 10 **Pages, Initial:** 2219 **final:** 2227 **Year:** 2016

Publication 12

Authors: Creus-Cuadros, A.; Tresserra-Rimbau, A.; Quifer-Rada, P.; Martínez-González, M.A.; Corella, D.; Salas-Salvadó, J.; Fitó, M.; Estruch, R.; Gómez-Gracia, E.; Lapetra, J.; Arós, F.; Fiol, M.; Ros, E.; Serra-Majem, L.; Pintó, X.; Moreno, J.J.; Ruiz-Canela, M.; Sorlí, J.V.; Basora, J.; Schröder, H.; Lamuela, R.M.

Title: Associations between Both Lignan and Yogurt Consumption and Cardiovascular Risk Parameters in an Elderly Population: Observations from a Cross-Sectional Approach in the PREDIMED Study

Journal: Journal of the Academy of Nutrition and Dietetics

Volume: S2212-2672 **Number:** 16 **Pages, Initial:** 31394 **final:** 31396 **Year:** 2016

Publication 13

Authors: Storniolo, C.E.; Quifer-Rada, P.; Lamuela-Raventos, R.M.; Moreno, J.J.

Title: Piceid presents antiproliferative effects in intestinal epithelial Caco-2 cells, effects unrelated to resveratrol release

Journal: Food & Function

Volume: 5 **Number:** 9 **Pages, Initial:** 2137 **final:** 2144 **Year:** 2014

Publication 14

Authors: Choy, Y.Y.; Quifer-Rada, P.; Holstege, D.M.; Frese, S.A.; Calvert, C.C.; Mills, D.A.; Lamuela-Raventos, R.M.; Waterhouse, A.L.

Title: Phenolic metabolites and substantial microbiome changes in pig feces by ingesting grape seed proanthocyanidins

Journal: Food & Function

Volume: 5 **Number:** 9 **Pages, Initial:** 2298 **final:** 2308 **Year:** 2014

Publication 15

Authors: Chiva-Blanch, G.; Condines, X.; Magraner, E.; Roth, I.; Valderas-Martínez, P.; Arranz, S.; Casas, R.; Martínez-Huélamo, M.; Vallverdú-Queralt, A.; Quifer-Rada, P.; Lamuela-Raventos, R.M.; Estruch, R.

Title: The non-alcoholic fraction of beer increases stromal cell derived factor 1 and the number of circulating endothelial progenitor cells in high-cardiovascular risk subjects: a randomized clinical trial

Journal: Atherosclerosis

Volume: 233 **Number:** 2 **Pages, Initial:** 518 **final:** 524 **Year:**

Publication 16

Authors: Di Lecce, G; Arranz, S; Jáuregui, O; Tresserra-Rimbau, A; Quifer-Rada, P; Lamuela-Raventós, R.M.

Title: Phenolic profiling of the skin, pulp and seeds of Albariño grapes using hybrid quadrupole time-of-flight and triple-quadrupole mass spectrometry

Journal: Food Chemistry

Volume: 145 **Number:** --- **Pages, Initial:** 874 **final:** 882 **Year:** 2014

8.2 Other book chapters

Authors: Waterhouse, A.L; Lamuela-Raventos, R.M; Quifer-Rada, P: Stocley, C.S

Title: Wine

Book: Handbook of functional beverages and human health

Publisher: Taylor and Francis

Volume: --- **Number:** --- **Pages, Initial:** 739 **final:** 755 **Year:** 2016

8.3 Conference communications

Authors: Choy, Y.Y; Mastaloudis, A; Wood, S.M; Hester, S.N; Gray, R; O'Donnell, R.P; Barger, J.L; Quifer-Rada, P; Lamuela-Raventós, R.M; Waterhouse, A.L

Title: Nutritional Polyphenol Metabolites' Kinetics in Plasma, Urine, and Saliva Following Consumption by Normal, Healthy, Subjects

Type of participation: Poster

Conference: Experimental Biology

Location: San Diego (UNITED STATES) **Year:** 2016

Authors: Creus-Cuadros, A.; Tresserra-Rimbau, A.; Quifer-Rada, P.; Martínez-González, M.A.; Corella, D.; Salas-Salvadó, J.; Fito, M.; Estruch, R.; Gómez-Gracia, E.; Lapetra, J.; Arós, F.; Fiol, M.; Ros, E.; Serra-Majem, L.; Pintó, X.; Ruiz-Canela, M.; Sorli, J.V.; Basora, J.; Schoeder, H.; Lamuela-Raventós, R.M.

Title: Yogurt and lignans work synergistically to improve cardiovascular risk parameters in an elderly population

Type of participation: Poster

Conference: XI International Conference on the Mediterranean Diet

Location: Barcelona (SPAIN) **Year:** 2016

Authors: Hurtado-Barroso, S.; Rinaldi-Alvarenga, J.F.; Quifer-Rada, P.; Creus-Cuadros, A.; Lamuela-Raventós, R.M.

Title: Diferencias nutricionales entre una dieta rica en Antioxidantes y una dieta baja en antioxidantes

Type of participation: Poster

Conference: XI International Conference on the Mediterranean Diet

Location: Barcelona (SPAIN) **Year:** 2016

Authors: Quifer-Rada, P.; Martínez-Las Heras, R.; Colmán-Martínez, M.; Callejón-Fernández, R.; Jimenez, B.; Lamuela-Raventós, R.M.

Title: El estrés hídrico y el cultivo ecológico condicionan los niveles de polifenoles del Aceite de oliva virgen

Type of participation: Poster

Conference: XI International Conference on the Mediterranean Diet

Location: Barcelona (SPAIN) **Year:** 2016

Authors: Quifer-Rada, P.; Chiva-Blanch, G.; Jauregui, O.; Estruch, R.; Lamuela-Raventos, R.M.

Title: Urine metabolome changes after Beer and non-alcoholic beer intake

Type of participation: Poster

Conference: II Workshop Anual sobre 'Cacao y chocolate; ciencia y gastronomía'

Location: Santa Coloma de Gramenet (SPAIN) **Year:** 2016

Authors: Tresserra-Rimbau, A.; Quifer-Rada, P.; Martínez-Huélamo, M.; Creus-Cuadros, A.; Sasot, G.; Colmán-Martínez, M.; Guo, X.; Lamuela-Raventós, R.M.

Title: Development of new biomarkers for nutritional epidemiology

Type of participation: Poster

Conference: Biomarkers and Health Claims on Food: BIOCLAIMS Meeting with stakeholders

Location: Palma de Mallorca (SPAIN) **Year:** 2015

Authors: Quifer-Rada, P.; Choy, Y.Y.; Lamuela-Raventós, R.M.; Calvert, C.C.; Waterhouse, A.L.

Title: Effect of Short-term intake of grape seed extract on fecal metabolome. A metabolomics approach in a pig model

Type of participation: Poster

Conference: Young Research Fellow Meeting 2015

Location: Romainville (FRANCE) **Year:** 2015

Authors: Creus-Cuadros, A.; Quifer-Rada, P.; Guo, X.; Colmán-Martínez, M.; Mercader-Martí, M.; Lamuela-Raventós, R.M.

Title: Use of High resolution mass spectrometry tools for the screening of the polyphenolic metabolic differences between wine and dealcoholized wine

Type of participation: Poster

Conference: Young Research Fellow Meeting 2015

Location: Romainville (FRANCE) **Year:** 2015

Authors: Orrego-Lagarón, N.; Escribano-Ferrer, E; Martínez-Huélamo, M.; Quifer, P; Lamuela-Raventós, R.

Title: Co-administration of naringenin and quercetin in mice: study on the effect in their absorption and metabolism

Type of participation: Poster

Conference: 7th International Conference on Polyphenosl and Health, Tours (France), 27-30 oct 2015

Location: Tours (FRANCE) **Year:** 2015

Authors: Quifer-Rada, P.; Chiva-Blanch, G.; Jauregui, O.; Estruch, R.; Lamuela-Raventos, R.M.

Title: A metabolomic approach to elucidate beer polyphenol effects in a human cross over intervention trail

Type of participation: Poster

Conference: 7th International Conference on Polyphenol and Health (ICPH 2015)

Location: Tours (FRANCE) **Year:** 2015

Awarded with "Young Investigator poster award"

Authors: Creus-Cuadros, A.; Tresserra-Rimbau, A.; Quifer-Rada, P.; Salas-Salvadó, J.; Martínez-González, M.A.; Corella, D.; Estruch, R.; Fitó, M.; Lamuela-Raventós, R.M.

Title: Yoghurts and lignans work synergistically decreasing LDL cholesterol

Type of participation: Poster

Conference: 7th International Conference on Polyphenols and Health (ICPH 2015)

Location: Tours (FRANCE) **Year:** 2015

Awarded with "Young Investigator poster award"

Authors: Martínez-Huélamo, M.; Quifer-Rada, P.; Valderas-Martínez, P.; Arranz-Martínez, S.; Estruch, R.; Lamuela-Raventós, R.M.

Title: Naringenin-O-glucuronide as a biomarker of tomato consumption

Type of participation: Poster

Conference: 7th International Conference on Polyphenols and Health (ICPH 2015)

Location: Tours (FRANCE) **Year:** 2015

Authors: Colmán-Martínez, M.; Quifer-Rada, P.; Martínez-Las Heras, R.; Callejón-Fernández, R.; Jiménez, B.; Lamuela-Raventós, R.M.

Title: Effect of irrigation, the cultivation method and the olive variety on the phenolic content of Olive Oils

Type of participation: Poster

Conference: 7th International Conference on Polyphenols and Health (ICPH 2015)

Location: Tours (FRANCE) **Year:** 2015

Authors: Quifer-Rada, P.; Martínez-Las Heras, R.; Colmán-Martínez, M.; Callejón-Fernández, R.; Jiménez, B.; Lamuela-Raventós, R.M.

Title: La variedad de oliva, el estrés y el cultivo ecológico condicionan los niveles de polifenoles del aceite de oliva virgen

Type of participation: oral communication

Conference: I Workshop Anual INSA-UB. El universo del aceite de oliva

Location: Barcelona (SPAIN) **Year:** 2015

Authors: Paola Quifer-Rada; Anna Vallverdu-Queralt; Miriam Martinez-Huelamo; Gemma Chiva-Blanch; Olga Jáuregui; Ramon Estruch; Rosa Lamuela-Raventos

Title: Phenolic profiling of spanish beers by high resolution mass spectrometry LC-ESI-LTQ-ORBITRAP-MS)

Type of participation: Poster

Conference: X CONGRESO DIETA MEDITERRÁNEA

Location: Barcelona (SPAIN) **Year:** 2014

Authors: Paola Quifer-Rada; Ying Yng Choy; Rosa M. Lamuela-Raventós; Christopher C. Calvert; Andrew L. Waterhouse

Title: Procyanidin from grape seed extract modifies fecal endogenous metabolome

Type of participation: Poster

Conference: Oxidants and antioxidants in Biology

Location: Davis (UNITED STATES) **Year:** 2014

Authors: Anna Creus-Cuadros; Paola Quifer-Rada; Guo Xiaohui; Mariel Colman; Mercè Mercader Martí; Rosa M. Lamuela-Raventós.

Title: Screening the polyphenolic metabolic differences between wine and dealcoholized wine in plasma by high resolution mass spectrometry

Type of participation: Poster

Conference: II Jornada de Recerca en Enologia i Viticultura a Catalunya

Location: Tarragona (SPAIN) **Year:** 2014

Authors: Anna Creus-Cuadros; Paola Quifer-Rada; Guo Xiaohui; Mariel Colman; Mercè Mercader Martí; Rosa M. Lamuela-Raventós.

Title: Screening the polyphenolic metabolic differences between wine and dealcoholized wine in plasma by high resolution mass spectrometry

Type of participation: Poster

Conference: II Jornada de Recerca en Enologia i Viticultura a Catalunya

Location: Tarragona (SPAIN) **Year:** 2014

Authors: Quifer Rada, P.; Martinez Huelamo, M.; Jauregui, O.; Chiva Blanch, G.; Estruch, R.; Lamuela Raventos, R.M.

Title: Analysis of Prenylflavanoids in biological samples requires neutral pH. Differences in urinary excretion of Isoxanthohumol according to Gender

Type of participation: Poster

Conference: Diet and Optimum Health Conference

Location: Corvallis, Oregon (UNITED STATES) **Year:** 2013

Authors: Paola Quifer-Rada, Miriam Martínez-Huélamo, Ramón Estruch, Rosa Mº Lamuela-Raventós

Title: Prenylflavanoids as new biomarker of beer consumption

Type of participation: Poster

Conference: IX Congreso Internacional de Barcelona sobre la Dieta Mediterránea

Location: Barcelona (SPAIN) **Year:** 2012

Authors: Paola Quifer-Rada, Anna Tresserra-Rimbau, Sara Arranz, Alexander Medina-Remón, Giuseppe Di Lecce, Nuria Tobella, Mireia Torres, Rosa Mª Lamuela-Raventós.

Title: Influencia del clima y de la sobremaduración de las uvas variedad Merlot en el perfil fenólico del vino.

Type of participation: Poster

Conference: XI Congreso Nacional de Investigación Enológica

Location: Jerez de la Frontera (SPAIN) **Year:** 2011

Authors: Arranz, S.; Incer, A.; Tedechi, I.; Di Lecce, G.; Tresserra-Rimbau, A.; Quifer-Rada, P.; Medina-Remón, A.; Tobella, N.; Torres, M.; Estruch, R.; Lamuela-Raventos, R.M.

Title: Taninos condensados y Polifenoles Hidrolizables en uvas tintas de variedad Cabernet Sauvignon: evolución según clima y grado de madurez

Type of participation: Poster

Conference: XI Congreso Nacional de Investigación Enológica

Location: Jerez de la Frontera (Cádiz) (SPAIN) **Year:** 2011

Authors: Valderas-Martínez, P.; Arranz, S.; Di Lecce, G.; Medina-Remón, A.; Tresserra-Rimbau, A.; Quifer-Rada, P.; Velàzquez, A.; Tubio, M.; Estruch, R.; Lamuela-Raventos, R.M.

Title: Contenido en polifenoles totales y proteínas en uvas de la variedad Albariño en diferentes zonas climáticas durante el proceso de maduración

Type of participation: Poster

Conference: XI Congreso Nacional de Investigación Enológica

Location: Jerez de la Frontera (Cádiz) (SPAIN) **Year:** 2011

Authors: Arranz, S.; di Lecce, G.; Treserra-Rimbau, A.; Quifer-Rada, P.; Medina-Remón, A.; Valderas-Martínez, P.; Tobella, N.; Torres, M.; Estruch, R.; Lamuela, R.M.

Title: Caracterización del perfil fenólico de la uva blanca de variedad Chardonnay mediante UHPLC-DAD acoplado a detector MS (LTQ Orbitrap)

Type of participation: Poster

Conference: XI Congreso Nacional de Investigación Enológica

Location: Jerez de la Frontera (Cádiz) (SPAIN) **Year:** 2011

Authors: Di Lecce, G.; Arranz, S.; Treserra-Rimbau, A.; Quifer-Rada, P.; Medina-Remón, A.; Lamuela-Raventos, R.M.

Title: Caracterización de compuestos fenólicos en piel, pulpa y semilla de uva Albariño por espectrometría de masas con q-TOF y triple cuadrupolo

Type of participation: Poster

Conference: XI Congreso Nacional de Investigación Enológica

Location: Jerez de la Frontera (Cádiz) (SPAIN) **Year:** 2011

Authors: Medina-Remón, A.; Arranz, S.; Tresserra-Rimbau, A.; Quifer-Rada, P.; Di Lecce, G.; Tobella, N.; Torres, M.; Lamuela-Raventos, R.M.

Title: Correlación entre compuestos polifenólicos identificados en vinos tintos y sus diferentes atributos de cata

Type of participation: Poster

Conference: XI Congreso Nacional de Investigación Enológica

Location: Jerez de la Frontera (Cádiz) (SPAIN) **Year:** 2011

Authors: Paola Quifer Rada, Miriam Martínez Huélamo, Ramon Estruch, Rosa Mª Lamuela Raventós

Title: Determination of prenylflavanoids in different spanish beers using liquid chromatography-mass spectrometry

Type of participation: Poster

Conference: 5ºth International Conference on Polyphenols and Health

Location: Sitges (SPAIN) **Year:** 2011

Authors: Giuseppe Di Lecce, Sara Arranz, Anna Tresserra Rimbau, Paola Quifer Rada, Alexander Medina Remon, Anna Velazquez, Miguel Tubio, Rosa Mª Lamuela Raventós.

Title: Wine phenolic markers identification by ultra fast chromatography coupled to high resolution mass spectometry.

Type of participation: Poster

Conference: 5ºth International Conference on Polyphenols and Health

Location: Sitges (SPAIN) **Year:** 2011

Authors: G. Di Lecce; A. Tressera-Rimbau; P. Quifer-Rada; C Andrés-Lacueva, R; Lamuela-Reventós.

Title: Effect of water availability in the process of phenolic maturity of the Albariño's grape.

Type of participation: Poster

Conference: VII Congreso ω-3, CLA y antioxidant

Location: Ancona (ITALY) **Year:** 2010

Authors: G. Di Lecce; A. Tresserra-Rimbau; P. Quifer-Rada; C. Andrés-Lacueva, R; Lamuela-Raventós.

Title: L'effetto della disponibilità di acqua nel processo di maturazione fenolica dell'uva Albariño.

Type of participation: Presentation of communication

Conference: VII Congreso ω-3, CLA y antioxidant

Location: Ancona (ITALY) **Year:** 2010

Authors: R. M. Lamuela_Raventos; A. Tresserra; P. Quifer; G. Di Lecce; A. Medina; S. Arranz; C. Andres-Lacueva; J. Coello.

Title: Resultados de polifenoles en la uva blanca línea troncal ensayos 2008 y 2009

Type of participation: Presentation of communication

Conference: 2nd International Conference on Organic and sustainable Wine Production and Climate –Change

Location: Vilafranca del Penedès (SPAIN) **Year:** 2010