Hull-less and coloured-grain barley as a source of valuable healthy bioactive compounds

Mariona Martínez Subirà

Hull-less and coloured-grain barley as a source of valuable healthy bioactive compounds

Doctoral Thesis

Mariona Martínez Subirà

Universitat de Lleida

TESI DOCTORAL

Hull-less and coloured-grain barley as a source of valuable healthy bioactive compounds

Mariona Martínez Subirà

Memòria presentada per optar al grau de Doctor per la Universitat de Lleida
Programa de Doctorat en Ciència i Tecnologia Agrària i Alimentària

Director/a
María de los Ángeles Moralejo Vidal
Maria Paz Romero Fabregat

Tutor/a
Maria Paz Romero Fabregat

2021

Preface

Dissertation presented by Mariona Martínez Subirà for the PhD degree from the University of Lleida. This thesis was carried out under the supervision of Marian Moralejo Vidal and Maria Paz Romero Fabregat in the Department of Crop and Forest Sciences. It is included in "Ciència i Tecnologia Agrària i Alimentària" doctorate program.

This thesis is part of the coordinated project entitled "Cebada para Usos Alternativos (CebUA)", AGL2015-69435-C3-1 from the Spanish Ministry of Economy, Industry and Competitiveness.

The PhD candidate was financed through a pre-doctoral fellowship "Formación de Personal Investigador" (BES-2016-078654) from the Spanish Ministry of Economy, Industry and Competitiveness (April 2017-September 2021).

PhD Candidate

Mariona Martínez Subirà

Director

María de los Ángeles Moralejo Vidal

Signature of approval

Co-Director

María Paz Romero Fabregat

Signature of approval

Acknowledgments

El meu agraïment en primer lloc és per les meves directores de tesi la Dra. Marian Moralejo i la Dra. Maria-Paz Romero pel seu excel·lent treball, per la seva disponibilitat, consells i suport constant, i per haver-me donat aquesta gran oportunitat en el món de la recerca. Igualment agraeixo al Dr. Ignacio Romagosa, l'investigador principal del projecte, per la seva excel·lent supervisió, assessorament constant i valuosos consells, així com els seus ànims i paciència durant tota la meva recerca doctoral. Ha estat un plaer poder treballar amb vosaltres i m'ha permès aprendre moltíssim.

Voldria agraïr especialment a la Dra. Roxana Savin per la seva ajuda amb els assajos a camp i per orientar-me en els moments de dubtes. Admiro la seva dedicació i entusiasme per la feina.

Moltes gràcies a l'Eva Puig per tota la seva ajuda amb la realització dels experiments i pel bon ambient de treball, gràcies per ajudar-me quan estava fins a dalt de feina i per donar-me suport quan estava baixa d'ànims.

També voldria agraïr a la Dra. Alba Macià per la seva ajuda amb els anàlisis de masses, una part clau en la realització d'aquesta tesi.

A totes aquelles persones dels departaments de Producció Vegetal i Ciències Forestals i de Tecnologia d'Aliments per ajudar-me i fer possible la realització d'aquest treball.

Agraïr també al Dr. Luis Cistué del Consejo Superior de Investigaciones Cientificas (CSIC), Estación Experimental de Aula Dei (EEAD) i al Dr. Patrick Hayes del Oregon State University a Corvallis, per ser els pares que van donar origen als genotips d'ordi alimentari utilitzats en aquesta tesi doctoral.

No em puc deixar a tota la gent de *Semillas Batlle, SA* a Bell-lloc d'Urgell, Lleida, agraïr especialment al Sr. Paco Batlle per proporcionar els genotips d'ordi alimentari que s'han utilitzat en aquest treball i al Sr. Rafa Costa pel seu suport durant els assajos a camp.

El meu agraïment a la Dra. Elena Albanell de la Universitat Autònoma de Barcelona per l'aprenentatge rebut en tècniques NIRS i pel seu suport amb la interpretació dels resultats obtinguts amb aquesta tècnica.

Agraïr també a la Dra. Ana Ferrer de l'Institut agroalimentari d'Aragó (IA2) de la Universitat de Saragossa per acollir-me en el seu departament i per la seva ajuda amb les mesures de l'índex glucèmic.

Voldria donar les gràcies al Sr. Santiago Romagosa per les excel·lents fotografies del gra i fraccions perlades d'ordi alimentari, i agraïr també a la María Goñi pel disseny d'algunes de les imatges esquematitzades que es mostren en aquesta tesi.

I would like to thank Dr. Bill Thomas for receiving me in his research group at the James Hutton Institute in Dundee, Scotland. My most sincere thanks to all the department colleagues, but specially to Dr. Kelly Houston for welcoming me and make my stay more enjoyable.

M'agradaria donar les gràcies a la Dra. Maria-José Motilva que em va donar la primera oportunitat de dedicar-me a la recerca i a qui mai estaré prou agraïda. Moltes gràcies a la Dra. Iziar Ludwig que em va acompanyar en els primers moments al laboratori molt abans de començar aquest camí.

Vull acabar dedicant unes línies a la meva família que des de l'inici sempre m'han animat i encoratjat per dur a terme aquest camí. Pare moltíssimes gràcies per tota la teva paciència i a tu Mare per la força que sempre em transmets, i com no podia ser d'una altra manera us vull dedicar aquest treball. Finalment, vull donar les gràcies al Marc per la seva comprensió, suport moral i per esperar-me durant aquest viatge en la investigació sense deixar de creure en mi.

Aquest treball és el resultat de l'esforç de tots vosaltres, moltíssimes gràcies per la vostra contribució i per dedicar-me el vostre temps durant tota la meva recerca doctoral.

Table of contents

Table of contents

List of figures

Chapter I

Chapter II

List of tables

List of tables

Summary

Barley was one of the most important food grains in the ancient world. However, barley consumption decreased over time due to the introduction of other cereals, particularly wheat and rye. Nowadays, there is an increasing trend to supply consumers with safe foods based on whole cereal grains and/or components with high nutritive value to promote health. Crops need to be more diverse in number and quality, more efficient, and allow for healthier food to be produced. In this context, barley is one of the cereals with the greatest genetic diversity and capacity to adapt to extreme environments. It is also receiving attention as a source of healthy food, since the nutritional properties of its grains are excellent due to the presence of nutrients and bioactive compounds. However, despite different international initiatives aimed at releasing new specific varieties for food, the amount of barley used for food, excluding the beverage sector, is currently negligible in most countries. The amount of nutrients and bioactive compounds in barley grain is strongly influence by genetic diversity, agronomic and environmental conditions. In this thesis, food barley genotypes with varied grain characteristics (number of rows, presence/absence of hulls, type of starch, grain colour) and an array of bioactive compounds, potentially susceptible to heat stress, were studied in the field under different temperature and environmental conditions. Thermal stress during grain filling affected grain weight and size and changed the relative composition of bioactive compounds. The relationship between heat stress and grain β-glucans and arabinoxylans content was indirect, as the resulting increases in concentrations of these were due to the lower grain weight under stress. On the contrary, heat stress had a significant direct impact on some phenolic compounds, increasing their concentrations differentially across genotypes, which contributed to an improvement in antioxidant capacity. Thus, our results supported growing food barley in high-temperature stress-prone areas, as some bioactive compounds and increased, as did the antioxidant capacity, regardless of the smaller grain size. The deposition patterns of bioactive compounds and antioxidant capacity were also established throughout the filling of the grain. The accumulation of dietary fibre followed grain weight. Anthocyanins followed a second-degree polynomial pattern, reaching a peak before grain maturation. Free and bound phenols decreased as the grain developed, suggesting that they are synthesized in early stages. The rate of deposition of bound phenols was more sensitive to genotypic changes. Antioxidant capacity decreased over time, the decay being less steep under stress for all genotypes. Hence, if a market develops for food-barley ingredients, early harvesting of non-mature grain should be considered as a way to maximize antioxidant capacity. Based on the spatial distribution of the bioactive compounds also studied in this thesis, the outermost 30% of the pearling fractions seems the best option for exploiting the antioxidant capacity of barley, whereas pearled grains could supply β-glucan enriched flours. Current regulations require the elimination of inedible husks from human foods. However, due to their high content in bioactive compounds and antioxidant capacity, they should be considered a valuable material for animal feed, particularly for ruminants. Differential pearling fractions from an array of genotypes were used to prepare special foods. Biscuits containing different proportions of purple barley ingredients (whole flour or fraction) were richer in bioactive compounds, had higher antioxidant capacity and lower estimated glycemic index than 100% refined and whole-wheat biscuits, with slight changes in physical properties. Baking did not affect β-glucans or arabinoxylans, favoured the release of phenolic compounds from the food matrix and increased antioxidant capacity. Barley anthocyanins are thermally unstable and exhibited high degradation rates, but were partially stabilized by tartaric acid. Hence, hull-less and purple-grain barley offers interesting new avenues to fulfil the increasing demand for healthy food products.

Resumen

La cebada era uno de los cereales alimenticios más importantes del mundo antiguo. Sin embargo, el consumo de cebada disminuyó a lo largo de la historia debido a la introducción de otros cereales, en particular el trigo y el centeno. En la actualidad, existe una tendencia creciente a brindar al consumidor alimentos seguros a base de cereales integrales y / o componentes con alto valor nutritivo para promover su salud. Los cultivos deben ser más diversos en número y calidad, más eficientes y permitir la producción de alimentos más saludables. En este contexto, la cebada es uno de los cereales con mayor diversidad genética y capacidad de adaptación a ambientes extremos. También está recibiendo atención como fuente de alimento saludable, ya que las propiedades nutricionales de sus granos son excelentes debido a la presencia de nutrientes y compuestos bioactivos. Sin embargo, a pesar de las diferentes iniciativas internacionales destinadas a lanzar nuevas variedades específicas para la alimentación, la cantidad de cebada utilizada como alimento, excluyendo el sector de bebidas, es actualmente casi insignificante en la mayoría de los países. La cantidad de nutrientes y compuestos bioactivos en el grano de cebada está fuertemente influenciada por la diversidad genética, las condiciones agronómicas y ambientales. En esta tesis se estudiaron en campo genotipos de cebada alimenticia con características de grano variadas (número de hileras, presencia / ausencia de cubiertas, tipo de almidón, color de grano) y una serie de compuestos bioactivos, potencialmente susceptibles al estrés por calor, bajo diferentes temperaturas y condiciones ambientales. El estrés térmico durante el llenado del grano afectó el peso y el tamaño del grano y cambió la composición relativa de los compuestos bioactivos. La relación entre el estrés por calor y el contenido de β-glucanos y arabinoxilanos del grano fue indirecta, ya que los incrementos resultantes en las concentraciones se debieron al menor peso del grano bajo estrés. Por el contrario, el estrés por calor tuvo un impacto directo significativo en algunos compuestos fenólicos, aumentando sus concentraciones de manera diferencial entre los genotipos, lo que contribuyó a una mejora en la capacidad antioxidante. Por lo tanto, nuestros resultados respaldaron el cultivo de cebada alimenticia en áreas propensas a estrés por altas temperaturas, ya que algunos compuestos bioactivos aumentaron, al igual que la capacidad antioxidante, independientemente del tamaño de grano más pequeño. También se establecieron patrones de deposición de compuestos bioactivos y capacidad antioxidante a lo largo del llenado del grano. La acumulación de fibra dietética siguió al peso del grano. Las antocianinas siguieron un patrón polinomial de segundo grado, alcanzando un pico máximo antes de la maduración del grano. Los fenoles libres y ligados disminuyeron a medida que se desarrollaba el grano, lo que sugiere que se sintetizan en las primeras etapas. La tasa de deposición de fenoles ligados fue más sensible a los cambios genotípicos. La capacidad antioxidante disminuyó con el tiempo, siendo la disminución menos pronunciada bajo estrés para todos los genotipos. Por lo tanto, si se desarrolla un mercado para los ingredientes alimenticios de cebada, la cosecha temprana de granos no maduros debe considerarse como una forma de maximizar la capacidad antioxidante. Basado en la distribución espacial de compuestos bioactivos también estudiados en esta tesis, el 30% más externo de las fracciones perladas parecen la mejor opción para explotar la capacidad antioxidante de la cebada, mientras que los granos perlados podrían suministrar harinas enriquecidas con β-glucanos. Las regulaciones actuales exigen la eliminación de las cubiertas no comestibles para la alimentación humana. Sin embargo, debido a su alto contenido en compuestos bioactivos y capacidad antioxidante, deben considerarse como un material valioso para la alimentación animal, especialmente para rumiantes. Se utilizaron fracciones perladas diferenciales de una variedad de genotipos para preparar alimentos especiales. Las galletas que contenían diferentes proporciones de ingredientes de cebada púrpura (harina integral o fracción) fueron más ricas en compuestos bioactivos, mostraron mayor capacidad antioxidante y menor índice glucémico estimado que las galletas 100% de trigo refinadas e integral, con ligeros cambios en las propiedades físicas. El horneado no afectó a los β-glucanos ni a los arabinoxilanos, favoreció la liberación de compuestos fenólicos de la matriz alimentaria y aumentó la capacidad antioxidante. Las antocianinas de cebada eran térmicamente inestables y exhibían altas tasas de degradación, pero fueron estabilizadas parcialmente por ácido tartárico. Por lo tanto, la cebada sin cubiertas y con granos morados ofrece nuevas e interesantes vías para satisfacer la creciente demanda de productos saludables.

Resum

L'ordi era un dels cereals alimentaris més importants del món antic. No obstant això, el consum d'ordi va disminuir al llarg de la història a causa de la introducció d'altres cereals, en particular el blat i el sègol. En l'actualitat, hi ha una tendència creixent a brindar al consumidor aliments segurs a base de cereals integrals i / o components amb alt valor nutritiu per promoure la seva salut. Els cultius han de ser més diversos en nombre i qualitat, més eficients i permetre la producció d'aliments més saludables. En aquest context, l'ordi és un dels cereals amb major diversitat genètica i capacitat d'adaptació a ambients extrems. També està rebent atenció com a font d'aliment saludable, ja que les propietats nutricionals dels seus grans són excel·lents a causa de la presència de nutrients i compostos bioactius. No obstant això, tot i les diferents iniciatives internacionals destinades a llançar noves varietats específiques per a l'alimentació, la quantitat d'ordi utilitzat com aliment, excloent el sector de begudes, és actualment gairebé insignificant en la majoria dels països. La quantitat de nutrients i compostos bioactius en el gra d'ordi està fortament influenciada per la diversitat genètica, les condicions agronòmiques i ambientals. En aquesta tesi es van estudiar en camp genotips d'ordi alimentari amb característiques de gra variades (nombre de fileres, presència / absència de cobertes, tipus de midó, color de gra) i una sèrie de compostos bioactius, potencialment susceptibles a l'estrès per calor, sota diferents temperatures i condicions ambientals. L'estrès tèrmic durant l'ompliment el gra va afectar el pes i la mida del gra i va canviar la composició relativa dels compostos bioactius. La relació entre l'estrès per calor i el contingut de β-glucans i arabinoxilans del gra va ser indirecta, ja que els increments resultants en les concentracions es van deure al menor pes del gra sota estrès. Per contra, l'estrès per calor va tenir un impacte directe significatiu en alguns compostos fenòlics, augmentant les seves concentracions de manera diferencial entre els genotips, el que va contribuir a una millora en la capacitat antioxidant. Per tant, els nostres resultats van donar suport al cultiu d'ordi alimentari en àrees propenses a estrès per altes temperatures, ja que alguns compostos bioactius van augmentar, igual que la capacitat antioxidant, independentment de la mida de gra més petit. També es van establir patrons de deposició de compostos bioactius i capacitat antioxidant al llarg de l'ompliment del gra. L'acumulació de fibra dietètica va seguir al pes del gra. Les antocianines van seguir un patró polinomial de segon grau, aconseguint un pic màxim abans de la maduració del gra. Els fenols lliures i lligats van disminuir a mesura que es desenvolupava el gra, el que suggereix que es sintetitzen en les primeres etapes. La taxa de deposició de fenols lligats va ser més sensible als canvis genotípics. La capacitat antioxidant va disminuir amb el temps, sent la disminució menys pronunciada sota estrès per a tots els genotips. Per tant, si es desenvolupa un mercat pels ingredients alimentaris d'ordi, s'ha de considerar la collita primerenca de grans no madurs per maximitzar la capacitat antioxidant. Basat en la distribució espacial de compostos bioactius també estudiats en aquesta tesi, el 30% més extern de les fraccions perlades semblen la millor opció per explotar la capacitat antioxidant de l'ordi, mentre que els grans perlats podrien subministrar farines enriquides amb β-glucans. Les regulacions actuals exigeixen l'eliminació de les cobertes no comestibles per a l'alimentació humana. No obstant això, a causa del seu alt contingut en compostos bioactius i capacitat antioxidant, s'han de considerar com un material valuós per a l'alimentació animal, especialment per a remugants. Es van utilitzar fraccions perlades diferencials d'una varietat de genotips per preparar aliments especials. Les galetes que contenien diferents proporcions d'ingredients d'ordi porpra (farina integral o fracció) van ser més riques en compostos bioactius, van mostrar major capacitat antioxidant i menor índex glucèmic estimat que les galetes 100% de blat refinat i integral, amb lleugers canvis en les propietats físiques. L'enfornat no va afectar als β-glucans ni als arabinoxilans, va afavorir l'alliberament de compostos fenòlics de la matriu alimentària i va augmentar la capacitat antioxidant. Les antocianines d'ordi eren tèrmicament inestables i exhibien altes taxes de degradació, però van ser estabilitzades parcialment per àcid tartàric. Per tant, l'ordi sense coberta i amb grans porpres ofereix noves i interessants vies per satisfer la creixent demanda de productes saludables.

Chapter I.

General introduction, main objectives and common methodological aspects

I.1. General introduction

This thesis is divided into seven chapters and one annex. The first chapter includes the general introduction, the main objectives and a brief description of the common methodological aspects (Chapter I). It is followed by four experimental chapters that represent the main objectives of this thesis and are based on articles that have been accepted by international journals (Chapters II, III, IV and V). The final chapters comprise the general discussion (Chapter VI) and the main conclusions of this thesis (Chapter VII). The Annex includes a paper also published in an international journal, a collaboration with the "Universitat Autònoma de Barcelona" for the development of a system based on instrumental NIR techniques for the detection of bioactive compounds in barley.

I.1.1. Brief history of barley

Barley (*Hordeum vulgare* L.) is one of the oldest and most widely grown cereals worldwide. Although its botanic origin is not fully known, the currently accepted theory is that barley was first domesticated from the wild species *Hordeum vulgare ssp. spontaneum* some 10,000 years ago in the Fertile Crescent in the Near East, which spans present-day Israel, northern Syria, southern Turkey, eastern Iraq and western areas of Iran. Barley seems to have been grown on a considerable scale in areas of Iraq and Jordan and from there, it spread to Egypt, Mesopotamia, north-western Europe and China following the trade routes (Newman and Newman, 2006; von Bothmer et al., 2003). In more recent times, barley has been widely cultivated all over the world.

Barley is one of the most geographically adaptable cereals. Historically, domesticated barley adapted to diverse local climatic conditions with varying rainfall, sunlight, temperature or soil characteristics. This subsequently led to a great diversity of landraces and modern varieties (Schmid et al., 2018). Currently, barley is one of the most important cereals, in fifth place in acreage and crop production worldwide, only exceeded by wheat, corn, rice and soybean. The global barley harvest was 156.41 million metric tons in the 2019/2020 crop year, increasing from around 140.6 million metric tons in 2018/2019 (Shahbandeh, 2020).

Barley has been used for beverages and food since ancient times. The Hebrews, Greeks and Romans used barley for food, as did much of Europe until the sixteenth century but its consumption then decreased due to the addition of other cereals for human nutrition, such as wheat, rice and corn, especially in the 19th and 20th centuries (Arendt et al., 2013). Nowadays, most barley production is used for animal feed (75–80% of global), malting (20–25%), human food (2–5%), and the remainder in the biofuel industry (Sullivan et al., 2013; Tricase et al., 2018). However, barley is still important as human food in some countries, particularly in Asia and North Africa.

Concerning Spain, barley arrived between 7650 and 7550 years ago by three possible routes: (1) over the Mediterranean to the coast of Valencia, (2) across the Pyrenees, and (3) from Morocco by sea to southern Spain (Moralejo et al., 1994; Martínez-Moreno et al., 2017). Currently, Spain is the seventh country in the world for total barley production after the Russia, France, Germany, Australia, Ukraine and Canada. Barley is the crop that occupies the largest agricultural area in Spain (7.9 Mt cultivated in 2.8 Mha), being mainly used for the production of feed (around 90%). Approximately 10% is dedicated to malting and a very small fraction for the production of human food, such as breakfast cereals or snacks (Romagosa, 2019).

I.1.2. Characteristics of barley

Barley is one of the cereals with the widest genetic diversity and capacity to adapt to extreme environments, since its range extends from the Equator to sub-Arctic regions and from sea level to the highlands of the Andes and the Himalayas. There are diverse types of barley classified by different criteria:

- Sowing season: barley is classified as winter, facultative or spring types. Winter barley is sown in late fall, while spring barley is sown in the spring to avoid damage caused by the cold of winter. Facultative barley can be sown in the spring or fall. Winter barleys carry vernalization and/or photoperiod sensitive genes whereas spring types do not. Thus, winter barleys are more cold tolerant than spring barleys.
- Row type: barley can be differentiated by the spike architecture (Figure I.1). A two-rowed barley has one fertile floret per rachis node, while a six-rowed barley has three fertile florets per rachis node (Newman and Newman, 2008).

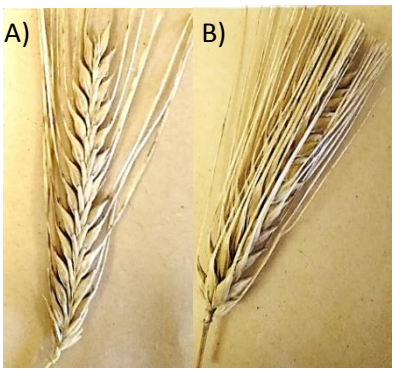


Figure I.1. **(A)** two-rowed barley and **(B)** six-rowed barley.

- Presence of husks: barley, contrary to wheat, have covered or hulled grains, with husks tightly adhering to the caryopsis. However, a few genotypes carry a mutation in the recessive ***nud*** gene on chromosome 7H that causes naked or hull-less grains (Figure I.2).

Figure I.2. (A) hull-less (naked) barley grains and **(B)** hulled barley grains.

- Grain colour: barley grain may develop different colours such as yellow, blue, red, purple, brown and black (Figure I.3). The different pigmentations are usually the results of phenolic compounds on the surface of the grain, particularly anthocyanins, proanthocyanidins or phytomelanins, that belong to the larger group of phytochemicals (Rybalka et al., 2020).

Figure I.3. Barley grains of different colours.

- Amylose content of the starch: three main barley types have been characterized regarding the amylose content of the starch: waxy (0-10%), normal (25%) and high amylose (>35%). The waxy gene, **wx**, is located on chromosome 7H and results in the elimination of the type-I granule bound starch synthase. The high amylose mutants carry the amo 1 locus located on chromosome 1H. The combination of waxy and high amylose genes can produce intermediate amylose contents of approximately 15% (Washington et al., 2000).
- End-uses: feed barley, used as animal feed and forage, malting barley, used for malt, food barley for human nutrition and the remainder for the biofuel industry.

I.1.3. Structure, composition and nutritional properties of the barley grains

The main parts of the grain are the husk, pericarp, testa, aleurone layer, endosperm, and embryo (Figure I.4). The husk and pericarp contain primarily cellulose, hemicellulose, lignin and lignans, the major constituents of insoluble fibre, but also minerals. The testa is composed of cellulose while the aleurone layer consists of protein-rich cells. The endosperm is a starchy mass in a protein matrix and the embryo is rich in proteins, lipids and ash (Izydorczyk and Dexter, 2004).

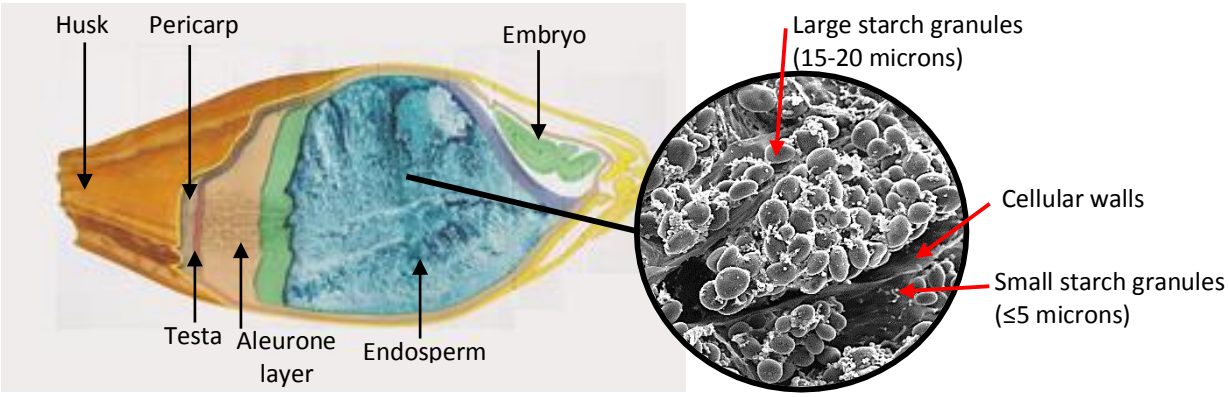


Figure I.4. The main parts of the barley grain (source adapted from Molina-Cano (1989)).

Furthermore, from a nutritional point of view, barley grain is valued for its high complex carbohydrate content, balanced protein level, low fat content, a good presence of vitamins, minerals, antioxidants and fibre (insoluble and soluble). Barley is also a good-source of bioactive compounds, components with potential health-promoting effects, such as β-glucans, arabinoxylans,

phenolic compounds, tocols, sterols and folates (Idehen et al., 2017). The most relevant bioactive compounds present in the barley grain for this work are the following:

- **β-glucans.** These are the major non-starch polysaccharides present in the cell walls of the barley grain. The structure of β-glucans is a linear chain polysaccharide made up of units of β-D-glucopyranosyl residues linked by 1, 3, and 1, 4 glycosidic bonds (Figure I.5). About 70% are located in the starchy endosperm cell walls while they make up 26% of the aleurone cell walls. The β-glucan contents of barley grain ranges from 4 to 9%. They are related to several positive health effects, such as maintaining normal blood cholesterol levels, reducing blood glucose after meals (FDA, 2008; EFSA, 2011a) and improving the responsiveness of the immune system against infectious diseases, inflammation and some types of cancer (Bashir and Choi, 2017).

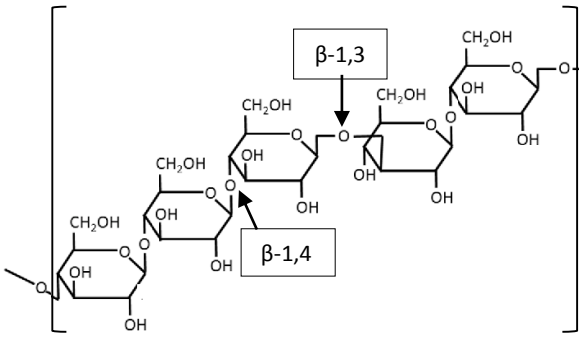


Figure I.5. General molecular structure of β-glucans in barley.

- **Arabinoxylans.** These constitute a fraction of dietary fibre which also have positive effects on the human digestive system. The structure of arabinoxylans comprises a linear xylose backbone with arabinose substitutions along the backbone (Figure I.6), and are the second most abundant barley cell wall polysaccharide. The arabinoxylan content of whole barley grains is about 4-7% by weight, being concentrated in the hulls and outer layers as aleurone cell walls (70%) and a lesser part in the starchy endosperm cell walls (20%). Arabinoxylans have been associated with a reduction of postprandial glycemic responses (EFSA,2011b) and other health-promoting properties, such as the nutritional benefits of soluble and insoluble fibre and antioxidant properties due to the presence of phenolic acids attached to their molecular structure (Izydorczyk and Dexter, 2008).

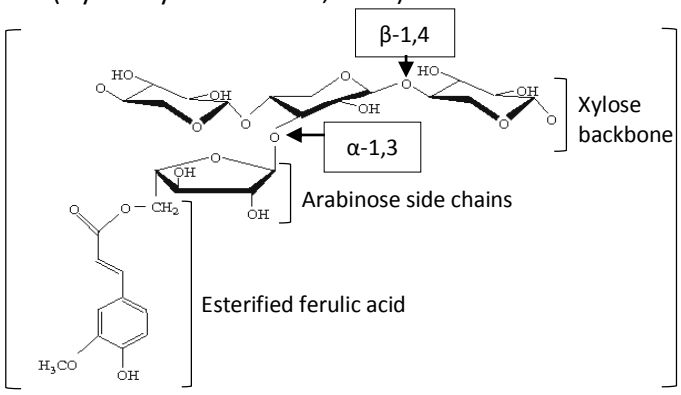


Figure I.6. General molecular structure of arabinoxylans with ferulic acid residue esterified to arabinose side chains in barley.

- **Tocols.** This is a class of lipid-soluble phytochemicals divided between tocopherols (T) and tocotrienols (T3). Barley grain contains α-, β-, γ- and δ-tocopherol and α-, β-, γ- and δ-tocotrienol in different proportions (Figure I.7). The tocols are composed of a chromanol ring with an attached phytyl C16 side chain. They can be distinguished by the number and location of the methyl groups on their chromanol ring: α (three methyl groups), β and γ (two methyl groups) and δ (one methyl group). Tocotrienols are mostly present in the endosperm and pericarp fraction whereas tocopherols are found in the germ. The total tocols in barley grain range from 46.7 to 67.6 mg/kg. Tocotrienols comprise 77% of the total tocols, the rest (23%) being tocopherols. Their biological activity is generally related to their antioxidant action where they inhibit lipid peroxidation in biological membranes. In addition to their antioxidant properties, tocols can confer such health benefits as modulating degenerative conditions like cancer and cardiovascular diseases (CVD) (Tiwari and Cummins, 2009). Studies also indicate that a high intake of α-tocopherols decreases platelet aggregation as well as functioning as a potent anti-inflammatory agent (Tiwari and Cummins, 2009; Jialal and Devaraj, 2005).

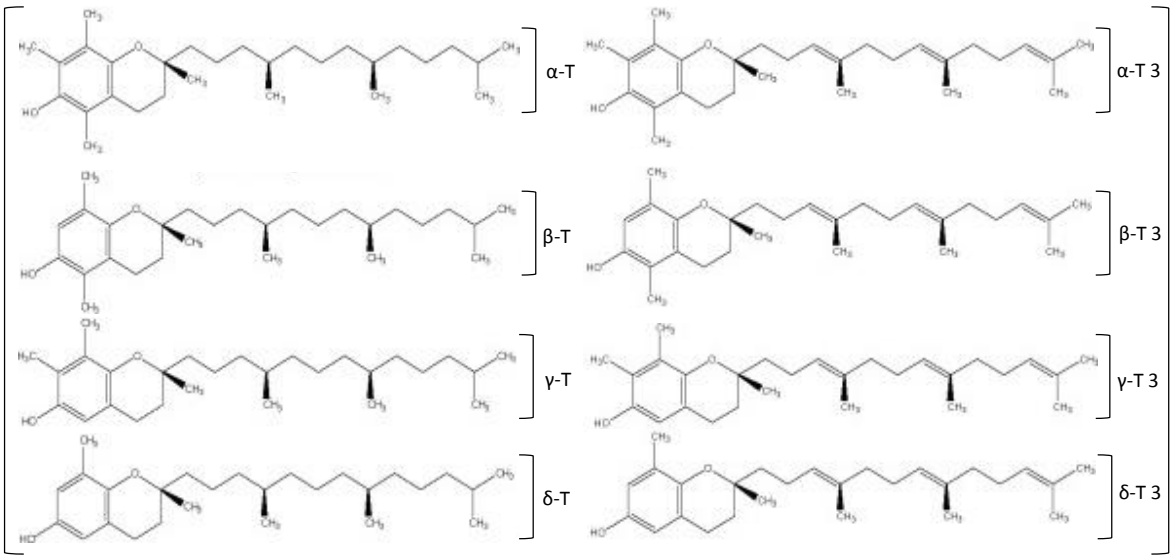


Figure I.7. The chemical structures of the tocopherols (T) and tocotrienols (T3) in barley.

- **Phenolic compounds.** These are secondary metabolites characterized by having at least one phenol unit that can be found free or bound to the fibre. Phenolic compounds are categorized into several major classes, including flavonoids and phenolic acids (Figure I.8). Flavan-3-ols, flavone glycosides and anthocyanins are the major types of flavonoids found mainly in the free phenolic fraction of barley grains, while phenolic acids are the most abundant in the bound phenolic fraction, particularly ferulic, p-coumaric (hydroxycinnamic) and vanillic (hydroxybenzoic) acids. Phenolic compounds are mainly located in the outer layers of the grain. For example, anthocyanins are located in the pericarp where they are responsible for the purple pigmentations or in the aleurone layer giving the grain a blue hue (Knievel et al., 2009). It has been described that the proanthocyanidins are found in the

testa layer, contributing to the yellow colour of the grain (Aastrup et al., 1984). The phenolic compounds possess antioxidant capacity and have been associated with a reduction of cardiovascular disease, inflammation and a range of cancers (Pandey and Rizvi, 2009).

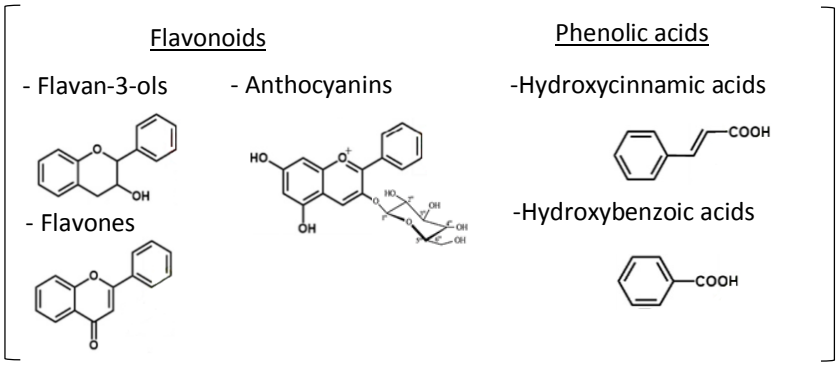


Figure I.8. Chemical structure of the main families of phenolic compounds in barley.

Despite the fact that barley possess great nutritional properties due to the presence of bioactive compounds, unevenly distributed among their grains, the amount of barley used for food excluding the beverage sector, is still very small. Furthermore, barley must undergo various processing steps before human consumption. These may affect its composition and physicochemical properties and compromise the health-promoting bioactive components in the grains (Sharma and Kotari, 2017). One primary physical processing method is pearling, an abrasive technique that gradually removes grain layers to obtain polished grain and by-products, allowing favourable separation of fractions enriched in specific compounds that can be used as functional ingredients (Figure I.9). For instance, barley flour made from polished grain has been used to produce bread, cakes, muffins, cookies, noodles and extruded snack foods (Tricase et al., 2018). A several fold enrichment of the level of antioxidant compounds in barley pearled fractions has been described (Irakli et al., 2020), and they have been used to improve the nutritional value of such wheat-based products as cookies, pasta and bread (Marconi et al., 2000; Sharma and Gujral, 2014; Blandino et al., 2015).

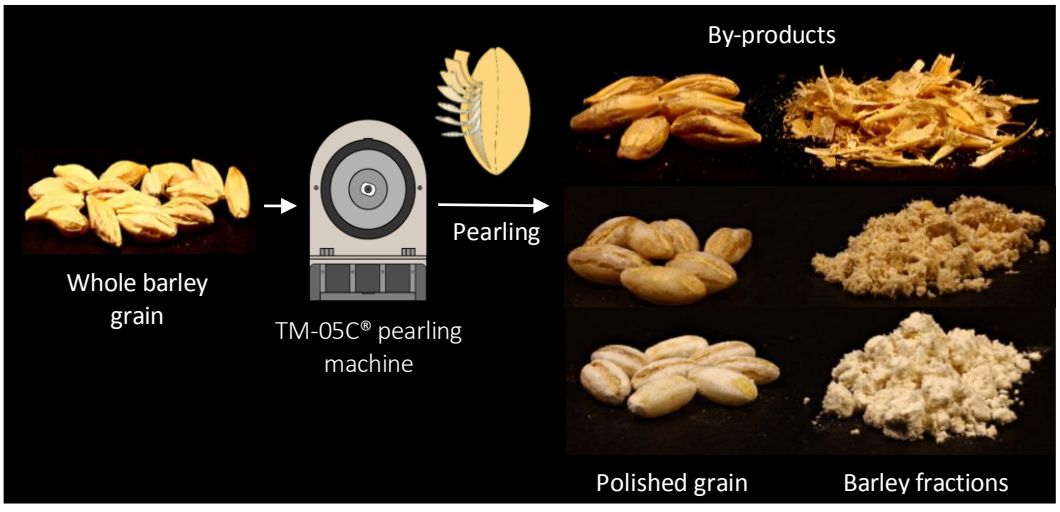


Figure I.9. Example of a pearling process, an abrasive technique that gradually removes grain layers to obtain polished grains and such by-products as barley fractions (source: personal author's elaboration).

I.1.4. Barley crop physiology

I.1.4.1. Crop development

The barley cycle can be divided into the following developmental stages: germination, seedling establishment and leaf and tiller differentiation, stem elongation, pollination (just before or during head emergence) and grain filling period.

The grain filling period begins after anthesis and fertilization. In this period, an accumulation of dry matter takes place that follows a sigmoid pattern (Dodig et al., 2018). The grain-filling period is characterized by three phases (Figure I.10): lag phase (fertilization and rapid cell division), the effective grain filling period (accumulation of reserve components), and the maturation drying phase (loss of water content and reaching "physiological maturity", i.e. maximum dry matter accumulation) (Loss et al., 1989; Egli, 2006).

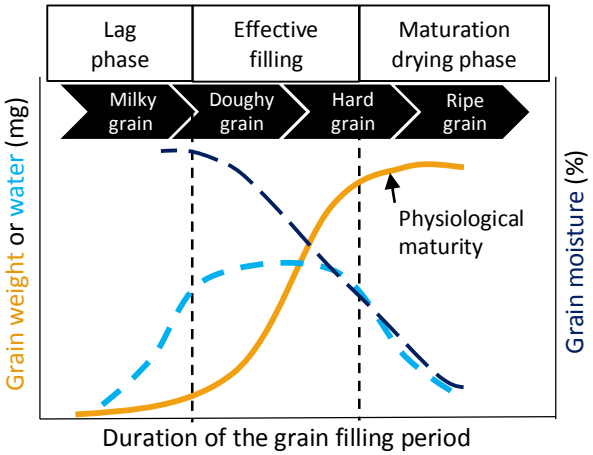


Figure I.10. Theoretical scheme showing the evolution of the grain weight, water content and moisture as a function of time expressed in days since the anthesis of the crop (source adapted from Miralles et al., 2014).

Both dry material and bioactive compounds accumulate in the effective grain filling phase (between the end of the lag phase and physiological maturity), determine the final weight, nutrient composition and quality of the grains (Schulman et al., 2000). Final grain size and weight are related to both the growth rate and the duration of grain filling. However, the relationship between the duration of filling and the rate of accumulation of the bioactive compounds present in the barley are still unclear. Insight into the time course and compositional changes of bioactive compounds during grain development is an important aspect for improving the nutritional quality of barley. Furthermore, identifying the timeframe when the maximum content of these bioactive compounds may occur could be useful for agronomic practices and also to promote further research into whether non-mature barley grain could be used as a functional ingredient in the elaboration of healthy cereal-based food products.

I.1.4.2. Temperature effects during grain filling and bioactive compound quality

The Intergovernmental Panel on Climate Change (IPCC) has projected that the global warming trend from 1986-2005 to 2081-2100 will show a temperature increase of between 0.3°C and 1.7°C (IPCC, 2013). Therefore, there are concerns about the impact of global climate change on the production

of crops like barley (Ko et al., 2019; Ezquer et al., 2020; Dell'Aversana et al., 2021). Future growing conditions will expose plants to variable and extreme climate change factors, with effects on the final bioactive compound contents (Ezquer et al., 2020). High maximum temperature during the grain filling period is one of the most relevant abiotic stresses under Mediterranean conditions. In fact, this situation is expected to occur more frequently due to climate change. The deleterious effects of high temperature on barley yield and quality are well documented in the literature. Higher temperatures during grain filling reduce grain weight in barley (Savin and Nicolas, 1996; Savin et al., 1996; Wallwork et al., 1998; Passarella et al., 2002), with a decrease of up to 30% depending on the cultivar, time of exposure and duration of the stress (Savin and Molina-Cano, 2002). However, the published works about the effect of heat stress on the bioactive compounds in barley are either very limited or their results are inconsistent. For instance, although the effect of high temperatures on the β-glucan content has been studied, the results are contradictory: while some studies reported an increase (Swanston et al., 1997), in others, the β-glucan levels were reduced (Wallwork et al., 1998) or not affected (Savin et al., 1996). On the other side, there are very few studies on the variability of the arabinoxylan contents due to environmental factors (Henry, 1986; Zang et al., 2013).

Regarding the phenolic compounds in barley, environmental conditions may have a significant impact on the total content and their antioxidant capacity (Rao et al., 2020). However, the few studies that have examined the variation in the phenolic contents of barley due to environmental conditions have either focused on different locations or year of growth (Narwal et al., 2016; Rao et al., 2020), rather than focusing on a specific environmental effects such as heat stress. The effects of heat stress depend on the time, duration and intensity of exposure of the genotypes to heat and this determines the impact on the final contents of bioactive compounds (Iqbal et al., 2017). Agronomic and environmental conditions during the barley growing cycle strongly influence grain yield and composition (Zhou et al., 2020) and, thus, the dynamics of the accumulation of these bioactive compounds under heat stress is another crucial aspect for enhancing the nutritional value of barley at harvest in order to produce grain with a certain composition to provide health and nutritional benefits.

I.1.5. A historical perspective of barley food

Barley has been used in the preparation of many traditional dishes. In fact, in the earliest remains from Mesopotamia and Egypt, barley is much more abundant than wheat, and the oldest literature suggests that former was more important than the latter for human consumption. For the Sumerians, barley had a mystical nature, as they had a goddess named *Nisaba* for barley, but none for wheat (Grando and Gomez Macpherson, 2005). Barley was used as a food source and for medicinal applications in ancient Egypt.

A review of the history of barley foods by Newman and Newman (2006) details traditional barley-based dishes and recipes. This describes that a popular food in ancient Greece were recipes for barley *plus,* a highly seasoned oily pasta mix. Among Greek food, a coarse milled barley bread called *krimnitas* or *chondrinos* and a hull-less barley biscuit called *paximadia* which had to be soaked before eating were also popular. These products were later mixed with wheat flour to decrease their hardness.

In ancient Rome, wheat bread was considered more nutritious and digestible, superior to that of barley. As in later cultures, the barley bread called *panus hordeaceus* was consumed predominantly by slaves and the poor. In fact, wealthy citizens used barley bread as plates or trays. However, Roman gladiators called *hordearii* or "barley men" consumed barley-based soups or porridge because they thought it gave them more strength and stamina than other foods (Newman and Newman, 2006).

In the Viking era, barley was also used as food, for example "ash bread" was prepared by baking barley dough in hot ashes. In Eastern Europe, a barley-based product called *ini* was traditional and it could be stored for many months. *Ini* was prepared by frying hull-less barley grains on a special brazier, then the grains were ground to be stored and before consumption they were mixed with water and salt. Hull-less barley was also used to make flat cakes and soups in those regions. On the islands off the north coast of Scotland, a variety of barley called bere (six-rowed landrace variety) was very popular for milling into flour to make bannocks, breads and pastries. Whole grains of bere were used to prepare barley broth by boiling beef and barley, adding vegetables and a little sugar. The bere barley was also used to make the Scottish *mashlum*, a mixture of peas, beans and barley, which was consumed by all classes until the end of the 18th century. Flatbreads, a type of barley bread which could be stored for long periods of time as staple foods, were common in Sweden. Barley grain and barley grits were also used in the preparation of soups, porridges, meat mixtures and sausages mixtures. A type of small loaf with barley called *bolon* or *boulon* was made in the mountainous regions of France (Newman and Newman, 2006).

Formerly, the Maghreb's original inhabitants, the Amazigh, used barley and acorn flour to make the famous couscous dish, although today it is customarily made from durum wheat. However, barley couscous is still popular in such Amazigh strongholds as the Atlas Mountains, which stretch for more than 1,500 miles through the Maghreb. In fact, food companies produce barley semolina for preparing couscous (Figure I.11).

Figure I.11. Brands that offer barley semolina for the preparation of couscous.

Barley was incorporated among the people of Ethiopia following cultural practices dating back thousands of years. Barley porridge and fermented and unfermented barley beverages were essential for rituals of prosperity, harvest and marriage (Newman and Newman, 2006). Today barley remains a staple food crop for many Ethiopians. In fact, barley producers in Ethiopia have given it the name *gebs ye ehil nigus*, which means that barley is the king of crops, due to its suitability for preparing many of the traditional Ethiopian dishes such as *ingera, kita, dabo, kolo, genfo, beso, chuko, shamet, tihlo, kinch* and *shorba* (Figure I.12) (Mohammed et al., 2016).

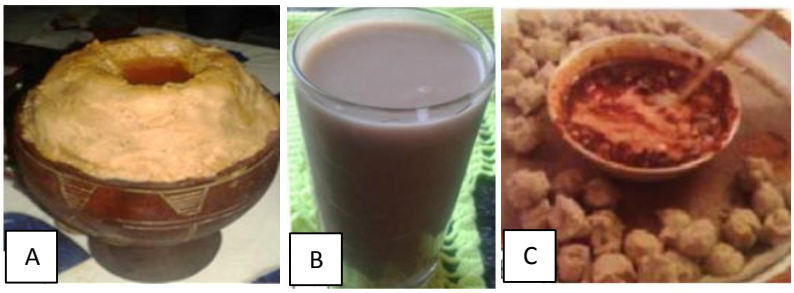


Figure I.12. Traditional Ethiopian barley dishes: **(A)** *Genfo* (barley porridge), **(B)** *Beso* in liquid form (mix of barley flour with cold water and sugar), and **(C)** *Tihlo* (a side dish, barley balls served with a hot sauce made from pulse flour and spices). (Source Mohammed et al., 2016).

The consumption of barley was also very popular in many parts of Asia, including Tibet, China, Japan, Korea and India. For example, the Tibetan diet included a barley porridge called *tsamba*. In it, the barley grains were roasted, ground into a very fine flour and made into flat cakes that were mixed with yak butter tea. Later in the south of Korea, barley was cultivated for many years as a rotational crop with rice. Although rice was the favorited cereal of Koreans, barley was used as an extender in many rice recipes. One of them was the *mixed bob*, prepared by cooking rice and precooked barley as a mixture (Newman and Newman, 2006). Barley has been grown in India since ancient times and is considered a sacred grain. It also has immense potential as a quality cereal especially from the nutritional and medicinal points of view. In India, its utilization as food crop is limited to the tribal hill areas. Such barley products as "*Sattu*" (because of its cooling effects on the human body in summer (Figure I.13)) and *missi roti* have been traditionally used in India (Kumar et al., 2014).

Figure I.13. Presentations of the barley product "*Sattu*" marketed in India as a natural energy drink, source of protein and fibre with health properties.

Currently, barley is important as human food in some regions including the Maghreb, Ethiopia, India or China, where it is still used in the preparation of many traditional dishes. Figure I.14 shows some food products or dishes made with barley flour.

Figure I.14. Food products or dishes made with barley: **(A)** coloured barley bread, **(B)** barley honey flatbread based on a traditional Viking recipe (source, Roots & Wren), **(C)** Algerian barley flatbread (source, The Teal Tadjine with Henia), **(D)** barley porridge with hulled barley, milk, unrefined sugar and fresh homemade butter (source, Ancient Food Culture), **(E)** sunflower barley crackers, **(F)** cheese, onion and bacon scones made with barley flour, **(G)** barley, beef and broccoli soup, and **(H)** beef and barley stew. (E, F, G and H images by Bryce Meyer).

I.2. Scope of the thesis

In recent years, increased efforts have been made in some Western countries to release new specific barley varieties for human consumption and the food industry. This doctoral thesis is embedded in a food barley breeding program, aimed at producing hull-less varieties rich in β-glucans and antioxidant compounds adapted to Spanish agro-climatic conditions. In this context, the general aims of the thesis are included in four activities carried out with food barley genotypes selected in the breeding program to:

(i) Determine the effect of thermal stress frequent under Mediterranean conditions on the final composition of the main bioactive compounds in fully mature barley grains (Chapter II);

(ii) Establish the deposition patterns of the main bioactive compounds and their antioxidant capacity throughout grain filling and analyse the effect of high temperatures on the accumulation of these bioactive compounds (Chapter III);

(iii) Determine the distribution of the main bioactive compounds in different barley pearling fractions and identify the best fractions to be used as functional ingredients (Chapter IV);

(iv) Assess the interest of these barley materials as ingredient in the development of new functional foods enriched in fibre and antioxidants for human consumption (Chapter V).

I.3. Common methodological aspects

The thesis includes field experiments and the use of commercial seed lots. The field experiments were carried out to induce heat stress on barley at Semillas Batlle, Bell-lloc d'Urgell (41°37'N, 0°47E), Lleida, Spain, under irrigated and well-fertilized conditions (Chapters II and III). Commercial seed lots produced also at Semillas Batlle were used to obtain the pearl fractions and in the development of functional foods (Chapters IV and V). The detailed descriptions of a particular experiment related to each chapter will be dealt with in the Material and Methods sections of respective chapter.

I.3.1. Plant Material

The barley varieties used in the following chapters differ in number of rows, presence/absence of hulls, type of starch, grain quality and colour.

- Annapurna®: registered Spanish variety, hull-less, two-rowed, waxy endosperm and high β-glucan content (semillas Batlle SA).
- Hindukusch: Afghan landrace, hull-less but often suffering from grain skinning, two rowed, non-waxy endosperm, purple grain and medium β-glucan content.
- Hispanic®: European variety, hulled, two-rowed, non-waxy endosperm and low β-glucan content.
- Kamalamai®: registered Spanish variety, hulled, two-rowed, non-waxy endosperm high β-glucan content (semillas Batlle SA).
- Tamalpais®: North American variety, hull-less grain, six-rowed, non-waxy endosperm and high β-glucan content.

I.3.2. Analytical techniques

A series of analytical techniques from basic enzymatic and colorimetric methods were used to measure β-glucans and arabinoxylans, along with advanced techniques such as high performance liquid chromatography (HPLC) and ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) for the quantification of tocols and phenolic compounds. Antioxidant capacity was measured with the oxygen radical absorbance capacity (ORAC) assay. Analytical techniques were also used to determine the estimated glycemic index (eGI) of barley-based foods through *in-vitro* digestibility and the kinetics of starch hydrolysis. Protein, ash and physical parameters were analysed with the official AACC and AOAC Official Methods.

I.4. References

Aastrup S, Outtrup H, Erdal K (1984) Location of the proanthocyanidins in the barley grain. Carlsberg Res. Commun. 49:105-109.

Ancient Food Culture. Barley porridge with hulled barley, milk, unrefined sugar and fresh homemade butter. Available: https://www.youtube.com/watch?v=oW0_WtzFIeg [26 March 2021].

Arendt E, Zannini E (2013) Cereal Grains for the Food and Beverage Industries. Philadelphia, Woodhead Publishing, p 512.

Bashir KM, Choi JS (2017) Clinical and physiological perspectives of β-glucans: The past, present, and future. Int. J. Mol. Sci. 18(9):1906.

Blandino M, Locatelli M, Sovrani V, Coïsson JD, Rolle L, Travaglia F, Giacosa S, Bordiga M, Scarpino V, Reyneri A, Arlorio M (2015) Progressive Pearling of Barley Kernel: Chemical

Characterization of Pearling Fractions and Effect of Their Inclusion on the Nutritional and Technological Properties of Wheat Bread. J. Agric. Food Chem. 63:5875-5884.

Bryce Meyer, excerpted from *Go Barley* by Pat Inglis and Linda Whitworth, copyright © 2014 by Pat Inglis and Linda Whitworth. Reprinted with permission of TouchWood Editions.

Dell'Aversana E, Hessini K, Ferchichi S, Fusco GM, Woodrow P, Ciarmiello LF, Abdelly C, Carillo P (2021) Salinity Duration Differently Modulates Physiological Parameters and Metabolites Profile in Roots of Two Contrasting Barley Genotypes. Plants 10:307.

Dodig D, Kandi V, Zori M, Nikoli-Dori E, Nikoli A, Mutavdži B, Perov D, Šurlan-Momirovi G (2018) Comparative kernel growth and yield components of two- and six-row barley (*Hordeum vulgare*) under terminal drought simulated by defoliation. Crop. Pasture Sci. 69:1215-1224.

EFSA (2011a) Scientific opinion on the substantiation of health claims related to β-glucans from oats and barley and maintenance of normal blood LDL-cholesterol concentrations (ID 1236, 1299), increase in satiety leading to a reduction in energy intake (ID 851, 852), reduction of post-prandial glycaemic responses (ID 821, 824), and "digestive function" (ID 850) pursuant to Article 13(1) of Regulation (EC) No 1924/2006. EFSA Journal, 2011,9 (6), 2207. Available: http://www.efsa.europa.eu/en/efsajournal/pub/2207 [5 June 2020].

EFSA (2011b) Scientific Opinion on the substantiation of health claims related to arabinoxylan produced from wheat endosperm and reduction of post-prandial glycaemic responses (ID 830) pursuant to Article 13(1) of Regulation (EC) No 1924/2006 Available: https://www.efsa.europa.eu/en/efsajournal/pub/2205 [5 June 2020].

Egli DB (2006) The role of seed in the determination of yield in grain crops. Aust. J. Agric. Res. 57:1237-1247.

Ezquer I, Salameh I, Colombo L, Kalaitzis P (2020) Plant Cell Walls Tackling Climate Change: Biotechnological Strategies to Improve Crop Adaptations and Photosynthesis in Response to Global Warming. Plants 9:212.

FDA (2008) Food Labeling: Health Claims; Soluble Dietary Fiber from Certain Foods and Coronary Heart Disease. [Docket No. FDA-2008-P0090]. Available: https://pubmed.ncbi.nlm.nih.gov/12361061/ [5 June 2020].

Grando S, Gomez Macpherson H (2005) Food Barley: Importance, Uses and Local Knowledge. S Grando, H Gomez Macpherson (Ed.), Proceedings of the International Workshop on Food Barley Improvement, 14-17 January 2002, Hammamet, Tunisia. ICARDA, Aleppo, Syria, pp.156.

Henry RJ (1986) Genetic and environmental variation in the pentosan and β-glucan contents of barley, and their relation to malting quality. J. Cereal Sci. 4:269-277.

Huang D, Ou B, Hampsch-Woodill M, Flanagan JA, Prior RL (2002) High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. J. Agric. Food Chem. 50:4437-4444.

Idehen E, Tang Y, Sang S (2017) Bioactive phytochemicals in barley. J. Food Drug Anal. 25:148-161.

IPCC (2013) Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. TF Stocker, D Qin, G-K Plattner, MMB Tignor, SK Allen, J Boschung, A Nauels, Y Xia, V Bex, PM Midgley (Ed.), Cambridge University Press: Cambridge, UK; New York, NY, USA, p. 1535.

Iqbal M, Raja NI, Yasmeen F, Hussain M, Ejaz M, Shah MA (2017) Impacts of Heat Stress on Wheat: A Critical Review. Adv. Crop Sci. Tech. 5:251.

Irakli M, Lazaridou A, Mylonas I, Biliaderis CG (2020) Bioactive Components and Antioxidant Activity Distribution in Pearling Fractions of Different Greek Barley Cultivars. Foods 9:783.

Izydorczyk MS, Dexter JE (2004) Barley: Milling and processing. In: C Wrigley, H Corke, C Walker (Ed.), Encyclopedia of Grain Science. Oxford, Elsevier; pp. 57-68.

Izydorczyk MS, Dexter JE (2008) Barley: β-glucans and arabinoxylans: Molecular structure, physicochemical properties, and uses in food products - a Review. Food Res. Int. 41: 850-868.

Jialal I, Devaraj S, (2005) Scientific evidence to support a vitamin E and heart disease health claim: research needs. J. Nutr. 135:348-353.

Knievel DC, Abdel-Aal E-S M, Rabalski I, Nakamura T, Hucl P (2009) Grain color development and the inheritance of high anthocyanin blue aleurone and purple pericarp in spring wheat (*Triticum aestivum* L.). J. Cereal Sci. 50(1):113-120.

Ko J, Ng CT, Jeong S, Kim J-H, Lee B, Kim H-Y (2019) Impacts of regional climate change on barley yield and its geographical variation in South Korea. Int. Agrophys. 33:81-96.

Kumar V, Khippal A, Singh J, Selvakumar R, Malik R, Kumar D, Kharub AS, Vermar RPS, Sharma I (2014) Barley (*Hordeum vulgare* L) research in India: Retrospect & Prospect. J. Wheat Res. 6(1):1-20.

Loss SP, Kirby EJM, Siddique KHM, Perry MW (1989) Grain growth and development of old and modern Australian wheats. Field Crop Res. 21:131-146.

Marconi E, Graziano M, Cubadda R (2000) Composition and Utilization of Barley Pearling By-Products for Making Functional Pastas Rich in Dietary Fiber and β-Glucans. Cereal Chem. J. 77:133-139.

Martínez-Moreno F, Igartua E, Solís I (2017) Barley types and varieties in Spain: A historical overview. Ciencia e Investigación Agraria 44 (1): 12-23.

Miralles DJ, González FG, Abeledo LG, Serrago RA, Alzueta I, García GA, de San Caledonio RP, Lo Valvo P (2014) "Manual de trigo y cebada para el cono sur procesos fisiológicos y bases de manejo". DJ Miralles, FG González, LG Abeledo, RA Serrago, I Alzueta, GA García, RP de San Caledonio, P Lo Valvo (Ed.). Orientación Gráfica Editora S.R.L. Buenos Aires, Argentina., p56.

Mohammed J, Seleshi S, Nega F, Lee M (2016) Revisit to Ethiopian traditional barley-based food. J Ethn Foods 3:135-141.

Molina-Cano JL (1989) LA CEBADA. Morfología, fisiología, genética, agronomía y usos industriales. Ministerio de Agricultura, Pesca y Alimentación. Servicio de Extensión Agraria. Ediciones Mundi-Prensa. Madrid, pp. 252.

Moralejo M, Romagosa I, Salcedo G, Sánchez-Monge R, Molina-Cano JL (1994). On the Origin of Spanish Two-Rowed Barleys. Theor. Appl. Genet. 87: 829-836.

Narwal S, Kumar D, Verma RPS (2016) Effect of Genotype, Environment and Malting on the Antioxidant Activity and Phenolic Content of Indian Barley. J Food Biochem. 40:91-99.

Newman CW, Newman RK (2006) A brief history of barley foods. Cereal Foods World 51:4-7.

Newman RK, Newman CW (2008) Barley: Taxonomy, Morphology and Anatomy. In: Barley for Food and Health: Science, Technology, and Products. John Wiley & Sons, Inc., pp. 18–31.

Pandey KB, Rizvi SI (2009) Plant polyphenols as dietary antioxidants in human health and disease. Oxid Med. Cell. Longev. 2:270-278.

Passarella VS, Savin R, Slafer GA (2002) Grain weight and malting quality in barley as affected by brief periods of increased spike temperature under field conditions. Aust. J. Agric. Res. 53:1-9.

Rao S, Santhakumar AB, Chinkwo KA, Blanchard CL (2020) Investigation of phenolic compounds with antioxidant activity in barley and oats affected by variation in growing location. Cereal Chem. 97:772–782.

Romagosa Clariana I (2019) La cebada mucho más que cerveza y pienso. Real academia de ingeniería.

Roots & Wren. Barley honey flatbreads based on a traditional Viking recipe. Available: http://rootsandwren.com/barley-honey-flat-breads/ [26 March 2021].

Rybalka OI, Morgun VV, Morgun BV (2020) Colored grain of wheat and barley — a new breeding strategy of crops with grain of high nutritional value. Fiziol. rast. genet. 52(2): 95-127.

Savin R, Molina-Cano JL (2002) Changes in malting quality and its determinants in response to abiotic stresses. In: GA Slafer, JL Molina-Cano, R Savin, JL Araus, I Romagosa (Ed.), Barley Science. Recent advances from molecular biology to agronomy of yield and quality. Food Product Press, New York, USA, pp.523-550.

Savin R, Nicolas ME (1996) Effects of short periods of drought and high temperature on grain growth and starch accumulation of two malting barley cultivars. Aust. J. Plant Physiology 23:201-210.

Savin R, Stone PJ, Nicolas ME (1996) Responses of grain growth and malting quality of barley to short periods of high temperature in field studies using portable chambers. Aust. J. Agric. Res. 47:465-477.

Schmid K, Kilian B, Russell J (2018) Barley domestication, adaptation and population genomics. In: N Stein, GJ Muehlbauer (Ed.), The barley genome. Springer, Cham, pp. 317–336.

Schulman AH, Runeberg-Roos P, Jääskeläinen M (2000) Grain filling and starch synthesis in barley. In: AK Gupta, N Kaur (Ed.), Carbohydrate Reserves in Plants-Synthesis and Regulation. Elsevier Science B.V.: Amsterdam, The Netherlands, pp. 147-167.

Shahbandeh, M. Barley production worldwide 2008/2009-2018/2019. (2020) Available: https://www.statista.com/statistics/271973/world-barley-production-since-2008/#statisticContainer [22 Mar 2021].

Sharma P, Gujral HS (2014) Cookie making behavior of wheat-barley flour blends and effects on antioxidant properties. LWT 55:301-307.

Sharma P, Kotari SL (2017) Barley: impact of processing on physico-chemical and thermal properties—a review. Food Rev. Int. 33:359-381.

Sullivan P, Arendt E, Gallagher E (2013) The increasing use of barley and barley by-products in the production of healthier baked goods. Trends Food Sci. Technol. 29:124-134.

Swanston JS, Ellis RP, Perez-Vendrell A, Voltas J, Molina-Cano JL (1997) Patterns of barley grain development in Spain and Scotland and their implications for malting quality. Cereal Chem. 74:456-461.

The Teal Tadjine with Henia. Algerian Barley Flatbread. Available: https://www.youtube.com/watch?v=C24tVrsmk6U [26 March 2021].

Tiwari U, Cummins E (2009) Nutritional importance and effect of processing on tocols in cereals. Trends Food Sci. Technol. 20:511-520.

Tricase C, Amicarelli V, Lamonaca E, Rana RL (2018) Economic analysis of the barley market and related uses. In: Z Tadele (Ed.), Grasses as Food and Feed. IntechOpen, London, UK. pp25-46.

von Bothmer R, Sato K, Komatsuda T, Yasuda S, Fischbeck G (2003) The domestication of cultivated barley. In: R von Bothmer, T van Hintum, H Knüppfer, K Sato (Ed.), Diversity in Barley (*Hordeum vulgare*). Elsevier Science B.V.; Amsterdam, The Netherlands, pp. 9-27.

Wallwork MAB, Logue SJ, MacLeod LC, Jenner CF (1998) Effect of high temperature during grain filling on starch synthesis in the developing barley grain. Aust. J. Plant Physiol. 25:173-181.

Washington JM, Box A, Karakousis A, Barr AR (2000). Developing waxy barley cultivars for food, feed and malt. Barley Genet. 8:303-306.

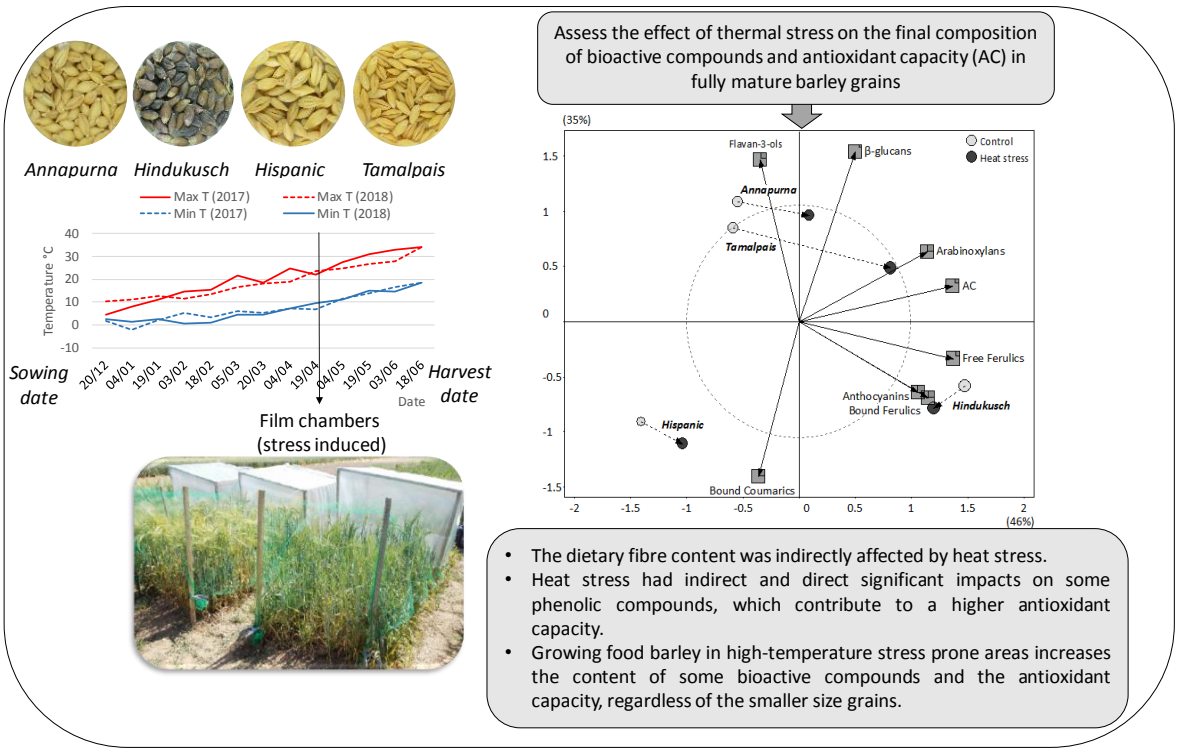
Zhang X, Wu F, Zhang G (2013) Genotypic and Environmental Variations of Arabinoxylan Content and Endoxylanase Activity in Barley Grains. J. Integr. Agric. 12(8): 1489-1494.

Zhou B, Jin Z, Schwarz P, Li Y (2020) Impact of Genotype, Environment, and Malting Conditions on the Antioxidant Activity and Phenolic Content in US Malting Barley. Fermentation 6:48.

Chapter II

Post-anthesis thermal stress induces differential accumulation of bioactive compounds in field-grown barley*

Mariona Martínez-Subirà, María-Paz Romero, Marian Moralejo, Alba Macià, Eva Puig, Roxana Savin, Ignacio Romagosa



Graphical abstract

* Martínez-Subirà, M.; Romero, M.-P.; Moralejo, M.; Macià, A.; Puig, E.; Savin, R.; Romagosa, I. Post-anthesis thermal stress induces differential accumulation of bioactive compounds in field-grown barley. J. Sci. Food Agric. published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry. 2021. https://doi.org/10.1002/jsfa.11321

Research Article

Received: 10 December 2020 Revised: 18 February 2021 Accepted article published: 17 May 2021 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/jsfa.11321

Post-anthesis thermal stress induces differential accumulation of bioactive compounds in field-grown barley

Mariona Martínez-Subirà, María-Paz Romero, Marian Moralejo, Alba Macià, Eva Puig, Roxana Savin and Ignacio Romagosa*

Abstract

BACKGROUND: Barley (*Hordeum vulgare* L.) is a healthy grain due to high contents in dietary fibre and phenolic compounds. It faces periods of high temperatures during grain filling, frequently reducing grain weight. Heat stress may also affect some of the bioactive compounds present in the grain. To produce quality grains that provide nutritional and health benefits, it is important to understand the effect of environmental stresses on the quantity and quality of bioactive compounds.

RESULTS: We have studied the effect of post-anthesis thermal stress on barley bioactive compounds and antioxidant capacity under Mediterranean field conditions during two consecutive growing seasons in four barley genotypes. Thermal stress affected grain weight and size and changed the relative composition of bioactive compounds. The relationship between heat stress and grain β-glucans and arabinoxylans content was indirect, as the resulting increases in concentrations were due to the lower grain weight under stress. Conversely, heat stress had a significant direct impact on some phenolic compounds, increasing their concentrations differentially across genotypes, which contributed to an improvement in antioxidant capacity of up to 30%.

CONCLUSION: Post-anthesis thermal stress had a significant effect on β-glucans, arabinoxylans, phenolic compound concentration and antioxidant capacity of barley grains. Final grain quality could, at least partially, be controlled in order to increase the bioactive concentrations in the barley grain, by cultivation in growing areas prone to heat stress. Late sowings or late flowering genotypes could also be considered, should a premium be implemented to compensate for lower yields.

Keywords: barley grain; thermal stress; dietary fibre, phenolic compounds, antioxidant capacity.

* Correspondence to: I Romagosa, University of Lleida – AGROTECNIO CERCA Center, Av. Rovira Roure 191, 25198 Lleida, Spain E-mail: ignacio.romagosa@udl.cat
University of Lleida – AGROTECNIO-CERCA Center, Lleida, Spain

II.1. Introduction

Barley (*Hordeum vulgare* L.) is the fourth most abundant cereal in the world being well adapted against extreme environmental conditions (FAOSTAT, 2019). Most barley is used for animal feed, about 6% for brewing malt and a less than 2% for food. Consumption is highest in Morocco with 20% of barley grain used in a variety of traditional dishes. Barley flour is increasingly used in some industrialized countries in new bread and pasta formulations, and whole grains, flours, differential pearling fractions and bioactive extracts are being evaluated to develop new food products, from non-alcoholic power drinks to meat-analogue burgers. Barley is a good source of bioactive compounds, components with potential health-promoting effects, such as β-glucans, arabinoxylans, phenolic compounds (PC), vitamin E (tocols), sterols and folates (Baik and Ullrich, 2008). β-Glucans and arabinoxylans are the major non-starch polysaccharides present in cell walls of the barley grain. β-Glucans are polymers of β-*D*-glucose with glycosidic linkages (1,4) and (1,3). They are related to several positives health effects, such as maintaining normal blood cholesterol levels, reduction of blood glucose after meals (FDA, 2008; EFSA, 2011a) and improving the responsiveness of the immune system against infectious diseases, inflammation and some types of cancer (Bashir and Choi, 2017). Arabinoxylans consists of (1,4)-β-linked xylopyranosyl residues, being the second most abundant barley cell wall polysaccharide. Arabinoxylans have been associated with reduction of postprandial glycemic responses (EFSA, 2011b) and other health-promoting properties, such as the nutritional benefits of soluble and insoluble fibre and antioxidant properties due to the presence of phenolic acids attached to its structure (Izydorczyk and Dexter, 2008). Barley is also a good source of PC, secondary metabolites characterized by having at least one phenol unit, that can be free or bound to the fibre. They possess antioxidant capacity and have been associated with the reduction of cardiovascular disease, inflammation and a diversity of cancers (Pandey and Rizvi, 2009).

Agronomic and environmental conditions during barley growing cycle strongly influence grain yield and grain composition (Rani et al., 2018; Zhou et al., 2020) and, thus, in order to produce grains with a certain composition to provide health and nutritional benefits, it is imperative to better understand the effect of environmental stresses on the quantity and quality of bioactive compounds. Deleterious effects of high temperature on barley yield and quality are well documented in the literature. For instance, it is well known that higher temperatures during grain filling reduce grain weight (GW) in barley in experiments performed under both controlled (Savin and Nicolas 1996; Wallwork et al., 1998) and field conditions, (Savin et al., 1996; Passarella et al., 2002), with a decrease in GW from 5 to 30% depending on the cultivar, time of exposure and duration of the stress (Savin and Molina-Cano, 2002). Furthermore, it is commonly accepted that accumulation of starch is more sensitive to high temperature than accumulation of nitrogen (Bhullar and Jenner, 1985; Jenner et al., 1991), as most of the experiments in barley when heat stresses was applied during grain filling period showed increases in grain nitrogen proportion when GW was reduced as a consequence of heat stress (Savin et al., 1996; Wallwork et al., 1998; Passarella et al., 2002). However, investigations on the impact of heat stress on grain bioactive compounds content are very limited. There are contradictory reports on the effect of high temperatures on the β-glucan content in barley: some studies reported an increase (Swanston et al., 1997), while others, barley β-glucan levels were reduced (Wallwork et al., 1998) or not affected (Savin et al., 1996). There have been very few studies on the variability of arabinoxylans content affected by environmental factors (Henry, 1986; Zhang et al., 2013) and none, which we know of, describing the effect of high-temperature stress on barley. Environmental conditions may also have a significant impact on total

phenolic content and antioxidant capacity in barley (Rao et al., 2020). Narwal et al. showed that the free phenol content was more influenced by the genotype, while the bound phenols were more influenced by the environment (Narwal et al., 2016). The few studies that have examined variation in phenolic content due to the environmental conditions on barley have either focused on different locations or year of growth (Narwal et al., 2016; Rao et al., 2020; Zhou et al., 2020) rather than focusing on a specific environmental effect such as heat stress. In addition, the effects of heat stress depend on the time, duration and intensity of exposure of the genotypes to heat (Iqbal et al., 2017) and this determines its impact on the final bioactive compounds content. Therefore, it is relevant to quantify the thermal stress effects on bioactive compounds under field conditions. Furthermore, in areas such as the Mediterranean basin, where high temperature stress is normally associated with the end of the growing season (Bavei et al., 2011), thermal stress is expected to be more frequent in the future (Li et al., 2013). Thus, the purpose of the current study was to investigate the effect of high temperatures from the mid-grain filling period to physiological maturity on GW, grain size, β-glucans, arabinoxylans, PC and their antioxidant capacity in four distinct barley genotypes under field conditions during two consecutive seasons.

II.2. Material and methods

II.2.1. Plant Materials and Treatments

Four barley genotypes were used in this study, differing in presence/absence of husks, number of rows, type of starch, grain quality and colour (Table II.S.1): Annapurna - two-rowed variety with hull-less (naked) grain, waxy endosperm and high β-glucan content; Hindukusch - Afghan two-rowed landrace with purple and partially hull-less grain, non-waxy endosperm and medium β-glucan content; Hispanic - two-rowed variety with hulled grain and non-waxy endosperm; Tamalpais - six-rowed variety with hull-less grain, non-waxy endosperm and high β-glucan content.

Heat stress was induced as described by Elía et al. (2018). Two temperature conditions were induced: a control and a high-temperature treatment, starting 15 days after heading (decimal code (Zadoks, 1974), DC55) and continuing up to physiological maturity (DC 90). The heat treatment was carried out by enclosing half of the plots with transparent polyethylene film (125 µm) mounted on wood structures 1.5 m in height above the soil level (Elía et al., 2018), but leaving the bottom 30 cm of the four sides of each structure open and punctures made in the top of the plastic to facilitate free gas exchange and reduce humidity. Stress increased maximum temperatures up to 8°C (Supporting Information, Table II.S.2), while the plastic cover reduced solar radiation by up to 15%. The two growing seasons differed significantly (Table II.S.2, Figure II.S.1). Spring 2017 was warmer (average 15°C *vs.* 13°C), drier (100 L m^{-2} *vs*. 175 L m^{-2} accumulated precipitation) and with higher solar radiation (+10 *vs.* -10 % long-term average); 2018 was warmer immediately after sowing (Servei Meteorologic de Catalunya 2017; 2018) Temperature were continuously registered from the start of the treatments during the two seasons (Table II.S.2). Average daily temperatures for the stressed and control treatments were 21.5 and 19.3°C and 20.6 and 18.0°C in 2017 and 2018, respectively. Average and maximum difference in thermal amplitudes under stress *vs*. control were 7.3 and 9.2°C in 2017 and 8.6 and 10.2°C in 2018. Temperatures under stress reached 45.1 and 44.4°C in 2017 and 2018, respectively. These extremely high temperatures are not that unusual at the end of grain filling under warm Mediterranean conditions.

II.2.2. Experimental Design

Fully irrigated and well-fertilized field experiments were conducted in Semillas Batlle, located in Bell-lloc d'Urgell (41°37'N, 0°47E), Lleida, Spain under irrigation and well-fertilized conditions. The sowing dates were 21 December 2016 and 20 December 2017, with rates of 350 seeds m^2. The main plot size was 4x1.8 m^2, from which two subplots of the same size were generated to apply the control treatment and the artificially induced continuous heat stress during grain filling.

II.2.3. Measurements and Analyses

II.2.3.1. Grain Weight and Grain Size

The barley grain was harvested at maturity, 45 days after anthesis (DAH). The spikes were threshed and cleaned with a LT-15 thresher (HALDRUP GmbH, Ilshofen, Germany) and samples individually packed and stored at -20°C until analysis. Grain weight was determined with a Marvin System according to the standard MSZ 6367/4-86 (1986) method. The grains were sieved using an electromagnetic sieve shaker (Filtra, Barcelona, Spain) to determine the percentage of grains retained through a nested slotted sieves of 2.2, 2.5 and 2.8 mm. Grain plumpness was estimated by the percentage weight of grains retained over a 2.5 mm sieve.

II.2.3.2. Milling

The barley seeds were milled using a Cyclotec 1093™ (FOSS, Barcelona, Spain) mill equipped with a 0.5 mm screen to produce whole meal flour, which was immediately kept at -20°C in the dark until analysis.

II.2.3.3. Quantitative Determination of β-glucans and Arabinoxylans

The total amount of mixed-linkage β-glucans and arabinoxylans in wholemeal flours were determined using the β-glucan assay (K-BGLU) and D-xylose assay (K-XYLOSE) kits from Megazyme (Wicklow, Ireland).

II.2.3.4. PC Extraction and Ultra-Performance Liquid Chromatographic-tandem Mass Spectrometric Analysis

Free and bound PC were extracted according to Martínez et al. (2018), subjected to a micro-elution solid-phase extraction (µSPE) (Waters, MA, USA) (Serra et al., 2013) and analysed by liquid chromatography (for more details see Martínez et al. (2018)). The phenolics were quantified by commercial reference to a 0.02–25 ng calibration curve of commercially available standard compounds and the results were expressed as micrograms per gram of dry sample. The limits of detection (LOD) ranged from 0.007 to 0.09 ng and limits of quantification (LOQ) from 0.02 to 0.30 ng. Total PC were calculated by adding all PC.

II.2.3.5. Determination of Antioxidant Capacity

The antioxidant capacity (AC) of the total PC in the barley grain was determined by the oxygen radical absorbance capacity (ORAC) assay according to Huang et al. (2002). The determination of ORAC was carried out using a FLUOstar OPTIMA fluorescence reader (BMG Labtech) in a 96-well polystyrene microplate controlled by OPTIMA 2.10R2 software, working at 485 nm for excitation and 520 nm for emission. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as control, with one ORAC unit being equal to the antioxidant protection given by 1 µmol Trolox. The antioxidant capacity of the extracts was calculated as µmols Trolox per gram of dry sample.

II.2.4. Statistical Analysis

Chemical determinations were carried out in triplicate and means used for the subsequent statistical analyses. A split-split-plot like-model with two full replicates was used, with year as main plots, genotypes in subplots and environment (heat stress and control) as sub-sub-plots. Two complementary statistical analyses were carried out. First, a direct standard analysis of variance (ANOVA) of the concentration (%) of every bioactive compound in the grain and an analysis of covariance (ANCOVA), using 1000-GW as the covariable, to elucidate whether possible alleged differences in concentration could be explained by changes in total GW. These analyses were conducted using restricted maximum likelihood (REML) mixed models in JMP®Pro 14 software (SAS institute Inc., Cary NC, USA), considering year, genotypes and environments fixed and block (year) and its interactions random.

II.3. Results and Discussion

The four genotypes studied differed widely in an array of bioactive compounds, potentially susceptible to heat stress from mid-grain filling to physiological maturity. However, we recognize that it may not be a representative set of food barley diversity.

II.3.1. Imposing High Temperatures Under Field Conditions

Experiments under controlled environment such as growth chambers are useful in understanding responses of plants to specific environmental factors, but they can differ considerably from field conditions and cannot be simply extrapolated to interpret variations in actual yield and quality observed under in the field (Passioura et al., 2020). In this study, high temperature was adequately and consistently imposed in the field with polyethylene film chambers (Supporting Information, Table II.S.2). However, reduced incident radiation (up to 15% at noon on very sunny days) was also registered. This reduction in incoming radiation did not significantly modify the source-sink balance for grain filling, as shown by Elía et al. (2018). The polyethylene film changed the partitioning of incoming radiation between direct and diffuse favouring the latter (Soar et al., 2009), which increased radiation use efficiency (Sinclair et al., 1992). Therefore, the reduction in incoming radiation was offset by an increase in radiation use efficiency.

II.3.2. Grain Weight (GW) and Grain Size

GW for the controls over the two growing seasons ranged from 44 to 52 mg across the four genotypes (Supporting Information, Table II.S.1). As expected, both environmental and genetic effects significantly influenced GW (Table II.1, Figure II.1A). Heat stress was the most important source of the differences, with control grains weighing on average 10% more than the stressed ones (Figure II.1A). The reduction of GW under heat stress from 15 days after heading to maturity was in agreement with previous studies on barley, which suggested that high temperature causes inactivation of sucrose synthase, leading to a reduction in the synthesis of starch that reduces the grain growth (Savin and Nicolas, 1996; Savin et al., 1996; Passarella et al., 2002; Rani et al., 2018). The difference in the average weight was reflected in grain size as grain plumpness was much lower under heat stress (Table II.1, Figure II.1B, Supporting Information Figure II.S.2), as also reported by Passarella et al. (2002). Genotypic differences were also significant, with Annapurna and Hispanic, both two-rowed commercial varieties, producing heavier and plumper grains than Hindukusch, a two-rowed landrace, and Tamalpais, a six-rowed cultivar.

Table II.1. Fixed-effect *F*-tests for the REML analyses of variance (ANOVA) of grain weight (GW) and grain plumpness (% grains >2.5mm) and of covariance (ANCOVA) for β-glucan and arabinoxylan concentrations in the grain, using GW as a covariable, of four barley genotypes grown under control and induced heat stress, 'Environment', for two consecutive years in Lleida, Spain.

Source	Grain Weight (mg)		Grain plumpness (%)		β-glucans (mg g^{-1})				Arabinoxylans (mg g^{-1})			
					ANOVA		ANCOVA		ANOVA		ANCOVA	
	F Ratio	p-value	F Ratio	p-value	F Ratio	p-value	F Ratio	p-value	F Ratio	p-value	F Ratio	p-value
GW							13.61	***0.0076***			3.23	*0.1195*
Year : [Y]	13.74	*0.0657*	0.03	*0.8819*	142.74	***0.0069***	1.83	*0.2072*	0.01	*0.9552*	0.09	*0.7644*
Genotype: [G]	10.66	***0.0081***	0.10	*0.9593*	75.31	***0.0000***	183.45	***0.0000***	16.81	***0.0025***	7.53	***0.0178***
G* Y	4.61	*0.0533*	0.43	*0.7364*	3.81	*0.0769*	75.59	***0.0000***	3.73	*0.0798*	6.86	***0.0232***
Environment: [E]	46.23	***0.0001***	4715.79	***0.0001***	4.84	*0.0590*	1.33	*0.2810*	12.51	***0.0076***	0.54	*0.4823*
Y *E	6.34	***0.0359***	50.16	***0.0001***	0.00	*0.9515*	3.76	*0.0782*	1.66	*0.2334*	0.22	*0.6529*
G *E	2.93	*0.0995*	21.95	***0.0001***	1.22	*0.3651*	1.98	*0.1803*	0.82	*0.5168*	0.28	*0.8352*
Y * G * E	2.21	*0.1647*	11.73	***0.0001***	3.88	*0.0556*	1.45	*0.2861*	0.07	*0.9756*	0.16	*0.9189*

Bold font indicates significant at *P* <0.05.

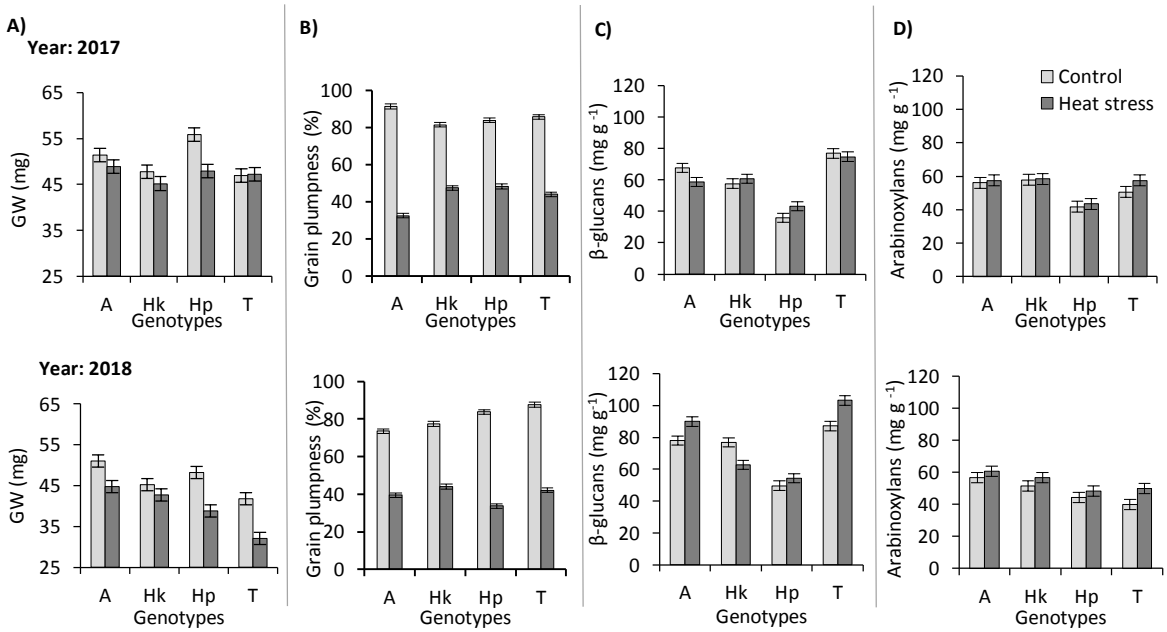


Figure II.1. (A) Average Grain Weight (GW) and **(B)** grain plumpness and ANCOVA least square means for **(C)** β-glucan and **(D)** Arabinoxylan concentrations in the grain of four barley genotypes (A: Annapurna; Hk: Hindukusch; Hp: Hispanic; T: Tamalpais) grown under control (light gray) and heat stress (dark gray) for two consecutive years in Lleida, Spain. Standard error presented by error bars. For statistical significance, see Table II.1.

II.3.3. Dietary Fiber

Genotype was the most important factor explaining the β-glucan content. This ranged from 80±2 mg g-1 in Tamalpais to 50±2 mg g-1 in Hispanic over the two years (Figure II.1C and Supporting Information, Table II.S.1). Although it has reported that waxy genotypes have higher β-glucans contents than non-waxy types (Izydorczyk and Dexter, 2008), the non-waxy genotype Tamalpais did not differ from the waxy genotype Annapurna. The grain β-glucan content was not significantly

altered by the continuous stress treatment (Table II.1, Figure II.1C). However, β-glucans were affected by annual variability, as the genotype × year interaction was statistically significant (Table II.1). β-Glucan levels were lower in 2017 (warm with higher solar radiation), than in 2018 especially for Annapurna and Tamalpais. There are contradictory reports on the effect of high temperatures on the β-glucan levels in barley grain (Savin et al., 1996; Swanston et al., 1997; Wallwork et al., 1998). Most of these studies were performed under controlled conditions, which are not easy to extrapolate to field conditions (Wallwork et al., 1998), and some of them study the heat stress by comparing different sites or sowing dates (Henry, 1986; Swanston et al., 1997), which have confounding effects. In our study, we did not detect any effect associated with the artificially imposed thermal stress but the year effect (annual variability) was highly significant, not interacting with any other term (Table II.1).

Arabinoxylans also varied among genotypes. Annapurna and Hindukusch had the highest average arabinoxylan content (55±3 mg g-1), followed by Tamalpais and Hispanic (Figure II.1D and Supporting Information, Table II.S.1). Although it has been suggested that six-rowed cultivars generally contain slightly higher levels of arabinoxylans than two-rowed genotypes (Izydorczyk and Dexter, 2008), in our study the highest arabinoxylan contents were observed in the two-rowed genotypes, Annapurna (waxy) and Hindukusch (non-waxy); presence of the waxy gene was not associated to a higher content of arabinoxylans as found by Izydorczyk and Dexter (2008). Arabinoxylan content could be also influenced by the environment (Henry, 1986; Zhang et al., 2013). Arabinoxylan content in wheat increased under high temperature stress (Rakszegi et al., 2014). Our results showed that the arabinoxylan concentration in the barley grain was apparently affected by thermal-induced stress; however, covariance analysis showed that any difference in arabinoxylans detected disappears once GW was introduced as covariable in the model (Table II.1). The apparently higher arabinoxylan concentration under stress could be explained by a concentration effect of the same amount of this pentosan in lighter grains, and not an apparent direct response to the induced heat stress. Although heat stress produced low flour yields due to thinner grains, the grains had dietary fiber concentrations equal or greater than under non-stressed conditions and thus enhanced healthy properties.

II.3.4. Antioxidant Capacity (AC)

Antioxidant capacity was significantly influenced by genotype and environment, both in the artificially induced stress and in year-to-year variation, either as main effects or at the level of some of their interactions (Table II.2, Figure II.2A). The highest antioxidant capacity was found in Hindukusch (140±7 μmols Trolox g-1) and the lowest in Hispanic (91±20 μmols Trolox g-1), in accordance with their total PC content (Figures II.2A, B and Supporting Information, Table II.S.1). These results were in line with those previously reported by Suriano et al. (2019), who found that grain of coloured barley genotypes had the highest antioxidant capacity and correlated significantly with their anthocyanin levels, as discussed below. Genotypes grown under heat stress had higher antioxidant capacity except for Hindukusch, which decreased from 143±4 to 126±4 μmols Trolox g-1. This reduction could be associated with a decrease in some PC, particularly anthocyanins due to use the polyethylene film as further discussed under ‘Anthocyanins’, below. The highest increase in antioxidant capacity due to stress was observed in Tamalpais (from 110±2 to 148±4 μmols Trolox g-1, i.e., 34%), and the lowest in Hispanic (91±9 to 107±9 μmols Trolox g-1, i.e., 18%).

Table II.2. Fixed-effect *F*-tests for the REML analyses of variance (ANOVA) and covariance (ANCOVA) for antioxidant capacity (AC) and total phenolic compounds (PC) concentrations in the grain, using GW as a covariable, of four barley genotypes grown under control and induced heat stress, 'Environment', for two consecutive years in Lleida, Spain.

Source	AC (µmols Trolox g^{-1})				PC (µg g^{-1})			
	ANOVA		ANCOVA		ANOVA		ANCOVA	
	F Ratio	p-value	F Ratio	p-value	F Ratio	p-value	F Ratio	p-value
GW			6.49	***0.0349***			19.01	***0.0024***
Year : [Y]	35.40	***0.0271***	11.38	***0.0064***	5.03	*0.1848*	3.70	*0.0863*
Genotype: [G]	91.50	***0.0000***	67.32	***0.0000***	17.82	***0.0028***	4.59	*0.0517*
G* Y	6.98	***0.0223***	15.95	***0.0019***	6.21	***0.0218***	11.53	***0.0060***
Environment: [E]	73.73	***0.0000***	1.20	*0.3019*	18.68	***0.0076***	7.87	***0.0227***
Y *E	1.14	*0.3167*	0.14	*0.7133*	0.16	*0.7090*	9.08	***0.0137***
G *E	13.73	***0.0016***	12.07	***0.0011***	2.20	*0.2063*	4.11	*0.0618*
Y * G * E	0.30	*0.8267*	6.96	***0.0080***	0.37	*0.7795*	3.58	*0.0773*

Bold font indicates significant at P <0.05.

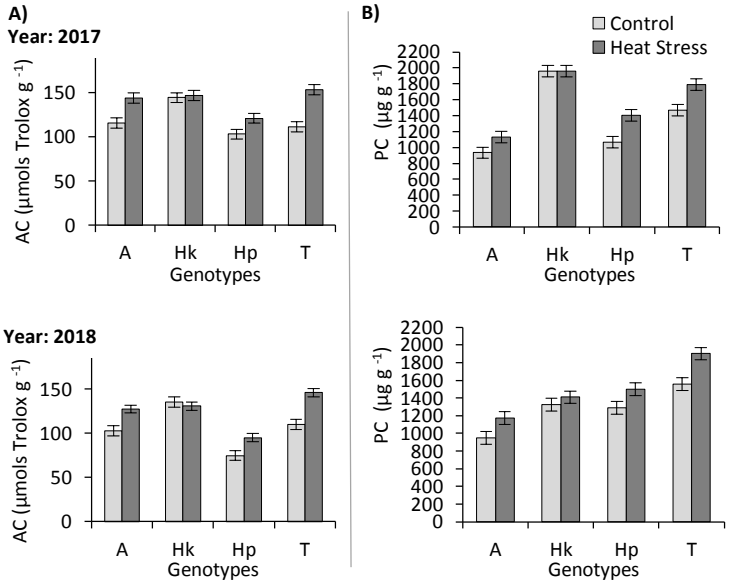


Figure II.2. ANCOVA least squares means for **(A)** antioxidant capacity (AC) and **(B)** total phenolic compounds (PC) in grain of four barley genotypes (A: Annapurna; Hk: Hindukusch; Hp: Hispanic; T: Tamalpais) grown under control (light gray) and heat stress (dark gray) for two consecutive years in Lleida, Spain. Standard error presented by error bars. For statistical significance, see Table II.2.

II.3.5. Total Phenolic Compounds (PC)

A total of 61 PC were identified in the four barley genotypes (Supporting Information, Table II.S.3). The 37 quantitatively most relevant - seven phenolic acids, nine flavan-3-ols and 21 anthocyanins - were selected to investigate the effect of high temperature. Phenolic acids detected in the free and bound fractions were ferulic and p-coumaric acids and their derivatives, representing an average of 72% of the total PC. The predominant flavan-3-ols were catechin and two dimers: procyanidin B3 and prodelphinidin B4 (average 77% of free fraction). The anthocyanin content was extremely high in the purple Hindukusch genotype, which was characterized by a high concentration of cyanidin-dimalonyl glucoside and cyanidin-glucoside (81% of the total anthocyanins).

Genotype was the most important factor in determining differences in total PC concentrations (Table II.2, Figure II.2B). Hindukusch and Tamalpais had the highest average content, 1649±450 and 1496±54 µg g-1 respectively (Supporting Information, Table II.S.1). This is in agreement to what has been reported, that purple (Kim et al., 2007) and six-rowed genotypes (Holtekjølen et al., 2006) had higher content of PC. Total PC was also affected by the thermal-induced stress, increasing content in all genotypes. However, significance decreased once the GW covariable was introduced in the ANCOVA model. This should be attributed to a dilution effect of PC in heavier non-stressed grains.

Year-to-year variability affected PC concentration more than artificially induced environmental changes. Weather conditions greatly influenced some PC accumulation in wheat varieties (Di Silvestro et al., 2017). However, differential behaviour of the four genotypes associated with annual variability was observed. PC contents for Annapurna and Tamalpais were apparently less affected by annual variability, while the PC concentration in Hindukusch markedly varied between different growing seasons. This could be related to grain pigmentation, because when Hindukusch was exposed to higher levels of solar radiation and grew under a warmer climate, such as that of 2017, grain pigmentation was more pronounced and concentrations of bound ferulic acid, flavon-3-ols and anthocyanins increased (Figure II.3). Therefore, these results suggested that climate had a deep impact on colour and the profile of PC in coloured barley grains.

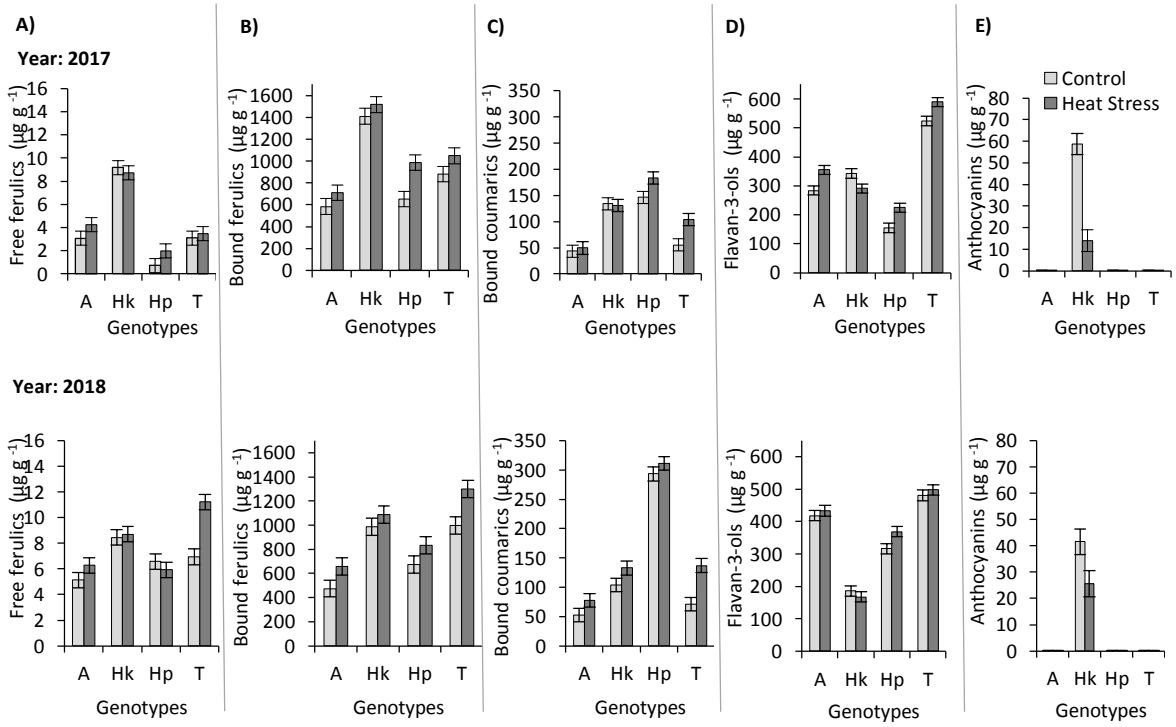


Figure II.3. ANCOVA least squares means for the main phenolic compounds concentration: **(A)** free ferulic acids; **(B)** bound ferulic acids; **(C)** bound coumaric acids; **(D)** flavan-3-ols and **(E)** anthocyanins in grain of four barley genotypes (A: Annapurna; Hk: Hindukusch; Hp: Hispanic; T: Tamalpais) grown under control (light gray) and heat stress (dark gray) for two consecutive years in Lleida, Spain. Standard error presented by error bars. For statistical significance, see Table II.3.

II.3.6. Phenolic Acids

There was not a common response in all four genotypes studied for all PC analysed; for a few of them the response to heat stress was genotypic dependent. The highest levels of free and bound ferulic acids were observed in the purple genotype 9±1 and 1246±37 µg g-1, respectively (Figures II.3 A, B). Conversely, Hispanic and Annapurna had the lowest, with 4±1, 5±1 µg g-1 and 786±64, 607±65 µg g-1, respectively. Hispanic (hulled genotype) had the highest coumaric acid concentration (233±17 µg g-1) while Annapurna had the lowest (56±17 µg g-1) (Figure II.3C). Our results agree with those of Holtekjølen et al. (2006) who observed higher content on coumaric acid in hulled barleys. Free and bound phenolic acids were indirectly associated with GW (Table II.3), suggesting that lower concentration in heavier grains (non-stressed) could be attributed to dilution effects. Phenolic acids differed across the genotypes, with lesser influence associated to stress. Conversely, bound coumaric acids differentially increased among genotypes (13-47%) under heat-induced stress. Previous research suggested that high temperature stress could influence the metabolic pathway of PC by increasing phenylalanine ammonia lyase activity, which catalyses the conversion of phenylalanine to trans-cinnamic acid, increasing the levels of some PC (Shamloo et al., 2017).

Table II.3. Fixed-effect *F*-tests for the REML analyses of covariance (ANCOVA) for main phenolic compounds concentration in the grain, using GW as a covariable, of four barley genotypes grown under control and induced heat stress, 'Environment', for two consecutive years in Lleida, Spain.

Source	Phenolic acids (µg g^{-1})						Flavan-3-ols (µg g^{-1})		Anthocyanins (µg g^{-1})	
	Free ferulics		Bound ferulics		Bound coumarics					
	F Ratio	p-value	F Ratio	p-value	F Ratio	p-value	F Ratio	p-value	F Ratio	p-value
GW	34.40	***0.0002***	23.54	***0.0019***	34.01	***0.0002***	2.10	*0.1969*	0.06	*0.8164*
Year : [Y]	0.05	*0.8337*	4.14	*0.1456*	3.05	*0.1066*	7.19	***0.0236***	0.13	*0.7288*
Genotype: [G]	11.76	***0.0037***	23.73	***0.0069***	25.07	***0.0001***	32.76	***0.0004***	27.51	***0.0003***
G* Y	14.84	***0.0017***	25.80	***0.0040***	9.68	***0.0042***	19.55	***0.0017***	14.87	***0.0015***
Environment: [E]	3.73	*0.0833*	2.46	*0.1657*	8.68	***0.0129***	3.47	*0.1055*	2.65	*0.1352*
Y *E	3.73	*0.0800*	3.82	*0.0786*	8.78	***0.0119***	5.59	***0.0460***	2.14	*0.1705*
G *E	3.40	*0.0697*	0.97	*0.4725*	5.59	***0.0249***	7.32	***0.0234***	7.96	***0.0067***
Y * G * E	1.90	*0.2005*	1.11	*0.4170*	4.68	***0.0378***	6.41	***0.0291***	9.56	***0.0034***

Bold font indicates significant at *P* <0.05.

II.3.7. Flavan-3-ols

The total flavan-3-ols were strongly affected by genotype and year x genotype interaction (Table II.3). The highest flavan-3-ol content was observed in Tamalpais (523±24 µg g-1), while the lowest was in Hindukusch (247±18 µg g-1) (Figure II.3D). The flavan-3-ol concentration varied between years. Tamalpais and Hindukusch had higher flavan-3-ols contents in 2017, marked by higher maximum temperatures and higher solar radiation during the grain filling period. In a previous study, we also found higher procyanidin C2 contents in barley samples grown in a warm environment than in a cool climate (Martínez et al., 2018). Therefore, warm climate could have a significant impact on the flavan-3-ol profile in barley.

Differential flavan-3-ol content of the genotypes was observed as response to environmental changes; it increased under heat stress in Annapurna (12%), Hispanic (23%) and Tamalpais (7%). To the best of our knowledge, the mechanism of flavan-3-ol synthesis upregulation in response to

abiotic stress, such as temperature and solar radiation, has not been fully elucidated in cereals. However, several studies have shown the influence of the environmental conditions on flavan-3-ol contents in other crops. Yao et al. showed in tea that the catechin contents were higher during warm months (Yao et al., 2005), while the catechin and proanthocyanidin contents were not greatly affected by partial exclusion of solar radiation in tea (Song et al., 2012) and grape berry (Martínez-Lüscher et al., 2017) or by UV-B radiation in apples (Lancaster et al., 2000). Although similar results had not been reported in cereals, variations in flavan-3-ol content could be more closely related to high-temperature stress than to changes in solar radiation.

II.3.8. Anthocyanins

Anthocyanins act as specific light protectors that absorb visible and UV radiations in vacuoles and prevent UV rays from penetrating into the tissue (Sharma et al., 2019). High anthocyanin content enhances absorption and tolerance to UV radiation as well as increasing its antioxidant capacity (Sharma et al., 2019). Therefore, blocking UV radiation with a conventional polyethylene film (Tsormpatsidis et al., 2008) may affect the accumulation of these compounds in the barley grain and, therefore, may reduce the antioxidant capacity. Differential genotypic responses associated with the pigmentation of the barley grain were observed for the anthocyanin content (Table II.3). The highest total anthocyanin contents were observed in Hindukusch (50±4 µg g-1), an old landrace collected from a high-altitude area, where protection from excess UV radiation is important. Concentrations for the other three yellow grain genotypes were extremely low: less than 0.8±4.7 µg g-1 (Figure II.3E). Anthocyanin concentration in Hindukusch under stress condition decreased 61% on average over the two years (Figure II.3E). Previous studies have suggested that UV radiation has a significant effect on anthocyanin accumulation. Blocking or decreasing UV radiation has been observed to reduce anthocyanin contents in strawberries (Josuttis et al., 2010) and apples (Henry-Kirk et al., 2018), while higher UV radiation levels increased the anthocyanin accumulation in purple wheat (Wang et al., 2019). Bustos et al. (2012) also observed a lower anthocyanin content in wheat grains from the shading of the spikes, proposing an effect of light on the genes controlling anthocyanin biosynthesis. These results reflect the influence of solar radiation on the accumulation of anthocyanins, suggesting that their decrease in Hindukusch was due to reduction of the incident radiation caused by the polyethylene film.

II.4. Conclusions

Heat stress during mid-grain filling period not only reduced final GW (on average by more than 10%) and size, but also changed the relative composition of its bioactive compounds. In the case of β-glucans and arabinoxylans, the relationship between heat stress and their content was indirect because the resulting increases in concentrations were due to the lower GW under stress. However, heat stress had indirect and direct significant impacts on some PC, increasing their concentrations differentially across genotypes (up to 20%). Grain under heat stress had more PC, which contribute to a higher antioxidant capacity of up to 30%, depending on the genotype. The lower incidence of solar radiation due to the use of conventional UV blocking polyethylene film reduced the anthocyanin accumulation in the purple grain genotype. Despite the influence of genotypic variations on the final grain quality, these findings highlight the importance of assessing the impact of heat stress periods on barley bioactive compounds, especially PC, to develop a better understanding of its subsequent impact on functional properties of these compounds for human

health. Future research would be necessary to determine whether the structure of some of these bioactive is affected by heat stress as it can influence the final quality of the barley-based product.

These findings support growing food barley in high-temperature stress-prone areas, as some bioactive compound and antioxidant capacity will increase, regardless the smaller size grains. Furthermore, if a market develops for food barley, late sowings or late flowering genotypes could also be recommended for any barley growing area, should a potential premium be implemented to compensate for the expected lower grains yield.

II.5. Funding

This work was funded by project AGL 2015-69435-C3-1 from the Spanish Ministry of Economy and Competitiveness. Mariona Martínez-Subirà was supported by a pre-doctoral fellowship (BES-2016-078654/AGL 2015-69435-C3-1).

II.6. Conflicts of interest

The authors declare no conflicts of interest.

II.7. Supporting information

Table II.S 1. Grain characteristics and average content of grain weight (GW), β-glucans, arabinoxylans, antioxidant capacity (AC) and total phenolic compounds (PC) of four barley genotypes during two consecutive seasons of the control samples without heat stress.

Genotypes		Endosperm	Hull	Row	Colour	β-glucan content	Average content of two growing seasons				
							GW (mg)	β-glucans (mg g $^{-1}$)	Arabinoxylan (mg g $^{-1}$)	AC (µmols Trolox g $^{-1}$)	PC (µg g $^{-1}$)
Annapurna	Spanish variety	Waxy	Naked	2	Yellow	High	51 ± 1 ^{a}	79 ± 7 ^{a}	55 ± 3 ^{a}	111 ± 9 ^{b}	994 ± 25 ^{b}
Hindukusch	Afghan landrace	Non-waxy	Partially naked	2	Purple	Medium	47 ± 2 ^{ab}	68 ± 1 ^{b}	55 ± 3 ^{a}	140 ± 7 ^{a}	1649 ± 450 ^{a}
Hispanic	European variety	Non-waxy	Hulled	2	Yellow	Low	52 ± 5 ^{a}	50 ± 2 ^{c}	41 ± 2 ^{c}	91 ± 20 ^{c}	1148 ± 164 ^{b}
Tamalpais	North American variety	Non-waxy	Naked	6	Yellow	High	44 ± 4 ^{b}	80 ± 2 ^{a}	45 ± 8 ^{b}	110 ± 1 ^{b}	1496 ± 54 ^{a}

Results are presented as mean ± SD. Mean with a column followed by different letters indicate significant differences; Tukey-Kramer (0.05).

Table II.S2. Daily temperature and thermal amplitude registered in artificially stressed and control plots during May 2017 and 2018

		Daily Temperature (°C)			Thermal amplitude (°C)		
Year	Environment	Mean	Max	Min	Mean	Max	Min
2017	Control	19.3	37.0	4.6	27.0	33.8	18.9
	Stressed	21.5	45.1	4.4	34.4	43.0	21.1
	Difference	*2.2*	*8.2*	*-0.1*	*7.3*	*9.2*	*2.2*
2018	Control	18.0	33.9	-0.4	25.6	33.2	18.2
	Stressed	20.6	44.4	-1.1	34.2	43.4	22.8
	Difference	*2.6*	*10.6*	*-0.6*	*8.6*	*10.2*	*4.6*

Table II.S3. Retention time (Rt), SRM and MS2 data of phenolic compounds identified in four barley genotypes

	Rt (min)	$[M-H]^{-/+}$ ($m\ z^{-1}$)	SRM transition [a]	Cone Voltage (V)	Collision Energy (eV)	MS2 fragments [b]	Quantified as	Quantification level [c]
Phenolic Acids and Aldehydes								
Gallic acid	1.54	169	125	30	20	125	3.4-Dihydroxybenzoic acid	Tent
2.4-Dihydroxybenzoic acid	2.77	153	109	45	15	109	3.4-Dihydroxybenzoic acid	Std
Caffeoyl-hexose	3.25	341	179	40	15	179, 135	Caffeic acid	Tent
p-Hydroxybenzoic acid	4.36	137	93	30	15	-	*p*-Hydroxybenzoic acid	Std
Sinapoyl-hexose	5.3	385	223	60	25	223, 205	Sinapic acid	Tent
Vanillic acid	5.59	167	123	30	10	152, 123	Vanillic acid	Std
Caffeic acid	5.84	179	135	20	12	135	Caffeic acid	Std
Syringic acid	6.33	197	182	30	10	182, 153	Syringic acid	Std
Feruloyl-pentose	7.57	325	193	40	10	193, 149, 134	Ferulic acid (*trans*)	Tent
p-Coumaric acid (*trans*)	8.8	163	119	35	10	119, 93	*p*-Coumaric acid (*trans*)	Std
Syringaldehyde	9.39	181	166	30	15	166, 151	Syringaldehyde	Std
Coumaric acid (*m*-Coumaric acid or *p*-Coumaric acid (*cis*))	10.28	163	119	35	10	119, 93	*p*-Coumaric acid (*trans*)	Tent
Ferulic acid (*trans*)	10.99	193	134	30	15	178, 149, 134	Ferulic acid (*trans*)	Std
Sinapic acid	11.68	223	164	35	15	208, 164, 149	Sinapic acid	Std
Hydroxybenzoic acid	12.41	137	93	30	15	93	*p*-hydroxybenzoic acid	Tent
Ferulic acid (*cis*)	12.81	193	134	30	15	178, 149, 134	Ferulic acid (*trans*)	Tent
Diferulic acid I	14.51	385	341	40	15	341, 326, 282, 297	Ferulic acid (*trans*)	Tent
Diferulic acid II	15.59	385	341	40	15	341, 326, 297, 282, 267	Ferulic acid (*trans*)	Tent
Diferulic acid III	17.86	385	341	40	15	341, 326, 282	Ferulic acid (trans)	Tent
Diferulic acid (tetrahydrofuran form)	18.37	403	282	40	15	282	Ferulic acid (*trans*)	Tent
Diferulic acid IV	18.77	385	341	40	15	341, 193, 178, 149, 134	Ferulic acid (*trans*)	Tent
Diferulic acid (decarboxylated form)	19.09	341	282	40	15	326, 282, 267, 297	Ferulic acid (*trans*)	Tent
Cinnamic acid	19.14	147	103	45	15	103	3.4-Dihydroxybenzoic acid	Tent
Triferulic acid I	19.38	577	355	50	20	533, 489, 355, 311, 193	Ferulic acid (*trans*)	Tent
Triferulic acid II	19.75	577	355	50	20	355, 193	Ferulic acid (*trans*)	Tent
Flavan-3-ols								
Catechin diglucoside	1.97	613	451	45	15	451, 289	Catechin	Tent
Prodelphinidin C2 (GC-GC-C)	2.4	897	593	60	25	593, 289	Catechin	Tent
Prodelphinidin B4 (GC-C)	2.78	593	289	45	20	289	Procyanidin B2	Tent
C-C-GC/GC-C-C	3.26	881	577	60	25	577, 289	Catechin	Tent
C-GC-C	3.48	881	593	60	25	593, 289	Catechin	Tent
Prodelphinidin B3 (GC-C)	4.21	593	289	45	20	467, 289	Procyanidin B2	Tent
Procyanidin B3 (C-C)	4.4	577	289	45	20	425 289	Procyanidin B2	Tent
Catechin-glucoside	4.6	451	289	45	15	289	Catechin	Tent
Procyanidin C2 (C-C-C)	4.8	865	289	60	30	577 289	Catechin	Tent
Catechin	5.01	289	245	45	15	245 205, 151	Catechin	Std
Procyanidin B2	6.31	577	289	45	20	289	Procyanidin B2	Std
Flavone glycosides								
Isoorientin (luteolin-6-**C**-glucoside)	10.95	447	357	50	20	-	Luteolin-7-*O*-glucoside	Tent
Isovitexin-7-*O*-rutinoside	11.2	739	431	60	20	431, 311	Apigenin-7-*O*-Glucoside	Tent
Isovitexin-7-*O*-(6-sinapoyl)-glucoside-4'-*O*-glucoside	11.34	961	799	60	20	799, 593	Apigenin-7-*O*-Glucoside	Tent
Apigenin-6-*C*-arabinoside-8-*C*-glucoside	11.35	563	353	60	20	473, 443, 383, 353	Apigenin-7-*O*-Glucoside	Tent
Isoscoparin-7-*O*-glucoside (chrysoeriol-6-*C*-glucoside)	11.57	623	341	60	20	623, 461, 341	Luteolin-7-*O*-glucoside	Tent
Isovitexin-7-*O*-(6-feruloyl)-glucoside-4'-*O*-glucoside	11.74	931	769	60	20	-	Apigenin-7-*O*-Glucoside	Tent
Isoscoparin-7-*O*-rutinoside	12.28	769	461	60	20	461, 341	Luteolin-7-*O*--glucoside	Tent
Luteolin-*O*-Glucoside	15.04	447	285	50	32	285, 133	Luteolin-7-*O*--glucoside	Std
Anthocyanins								
Delphinidin glucoside	1.38	465	303	40	20	-	Delphinidin-3-*O*-glucoside chloride	Std

Cyanidin glucoside	1.92	449	287	40	20	-	Cyanidin-3-*O*-glucoside chloride	Std
Petunidin-hexoside-hexoside	2.6	641	317	40	20	-	Malvidin-3-*O*-glucoside chloride	Tent
Pelargonidin-glucoside	2.7	433	271	40	20	-	Pelargonidin-3-*O*-glucoside chloride	Std
Malvidin-glucoside	3.3	493	331	40	20	-	Malvidin-3-*O*-glucoside chloride	Std
Delphinidin-dimalonylglucoside	3.4	637	303	40	20	-	Delphinidin-3-*O*-glucoside chloride	Tent
Carboxypyrano pelargonidin-glucoside	3.5	501	339	40	20	-	Pelargonidin-3-*O*-glucoside chloride	Tent
Petunidin-malonylglucoside	3.55	565	317	40	20	-	Delphinidin-3-*O*-glucoside chloride	Tent
Cyanidin-malonylglucoside	3.6	535	287	40	15	-	Cyanidin-3-*O*-glucoside chloride	Tent
Cyanidin-acetylglucoside	3.8	491	287	40	20	-	Cyanidin-3-*O*-glucoside chloride	Tent
Malvidin-hexoside-hexoside	4.14	655	331	40	15	-	Malvidin-3-*O*-glucoside chloride	Tent
Pelargonidin-acetylglucoside	4.34	475	271	40	20	-	Pelargonidin-3-*O*-glucoside chloride	Tent
Pelargonidin-malonylglucoside	4.36	519	271	40	25	-	Pelargonidin-3-*O*-glucoside chloride	Tent
Cyanidin-dimalonylglucoside	4.6	621	287	40	20	-	Cyanidin-3-*O*-glucoside chloride	Tent
Peonidin-malonylglucoside	4.7	549	301	40	20	-	Cyanidin-3-*O*-glucoside chloride	Tent
Delphinidin-malonylglucoside	4.9	551	303	40	20	-	Delphinidin-3-*O*-glucoside chloride	Tent
Malvidin-malonylglucoside	5	579	331	40	20	-	Malvidin-3-*O*-glucoside chloride	Tent
Peonidin-acetylglucoside	5	505	301	40	20	-	Cyanidin-3-*O*-glucoside chloride	Tent
Pelargonidin-dimalonylglucoside	5.43	605	271	40	20	-	Pelargonidin-3-*O*-glucoside chloride	Tent
Peonidin-dimalonylglucoside	5.6	635	301	40	20	-	Cyanidin-3-*O*-glucoside chloride	Tent
Petunidin-rutinoside	6.1	625	317	40	15	-	Malvidin-3-*O*-glucoside chloride	Tent

[a] SRM transition: single reaction monitoring used for quantification. [b] MS2 fragment: fragmentation products used for identification. [c] Std: Standard in which the phenolic has been quantified. When the phenolic was not quantified with its own standard, the quantification was tentative (Tent).

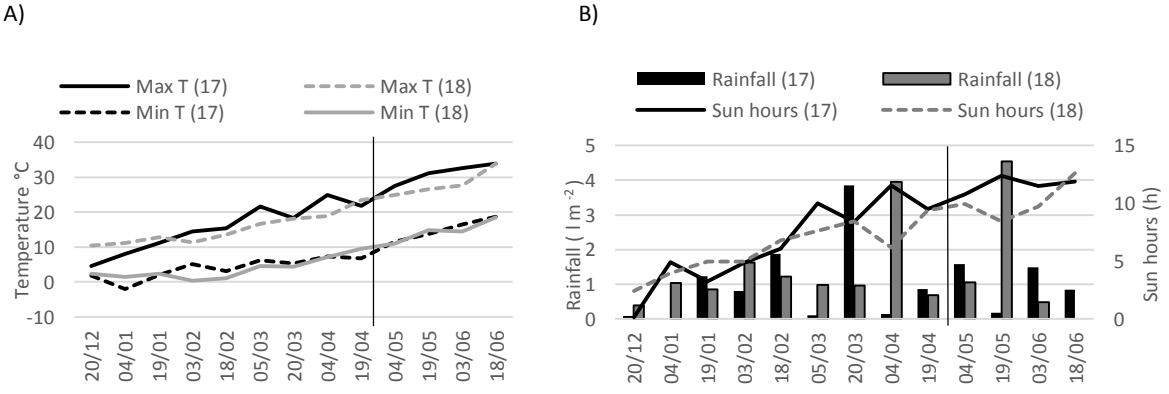


Figure II.S.1. A) maximum and minimum temperatures and B) rainfall and sun hours from sowing date to harvest time of two seasons 2016-2017(17) and 2017-2018 (18). The narrow vertical line marks the beginning of the treatment with the plastic cover.

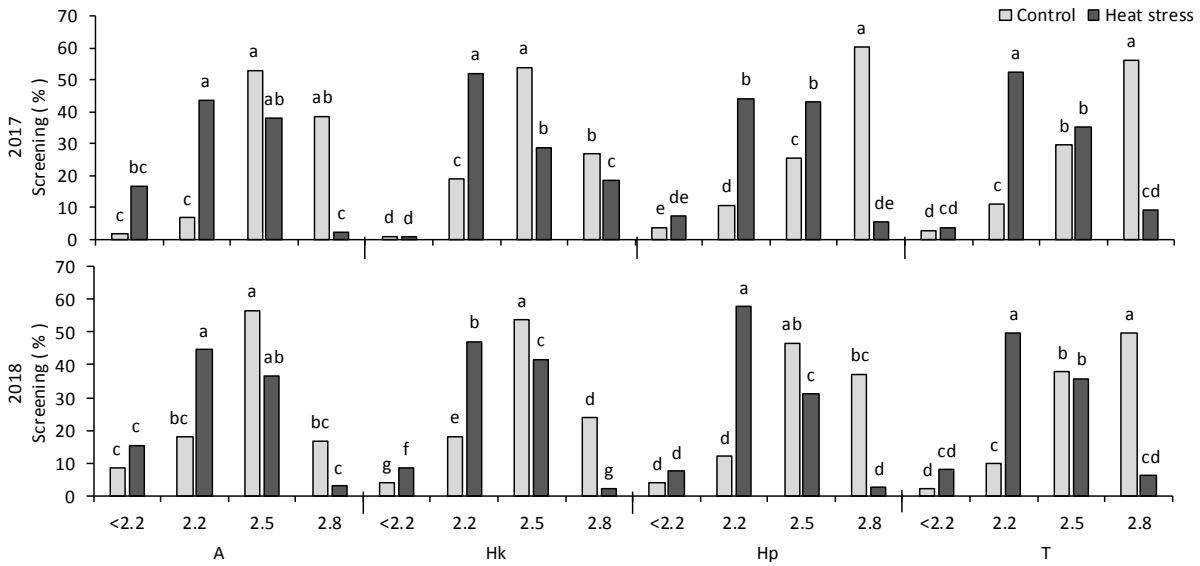


Figure II. S.2. Average percentage weight of grains retained through a nested slotted sieves of 2.2, 2.5 and 2.8 mm of four barley genotypes (A: Annapurna; Hk: Hindukusch; Hp: Hispanic; T: Tamalpais) grown under control (light gray) and heat stress conditions (dark gray) during two consecutive years. Different letters indicate significant differences within screening, genotype and year; (Tukey-Kramer HSD, α= 0.05).

II.8. References

Baik BK, Ullrich SE (2008) Barley for food: Characteristics, improvement, and renewed interest. J Cereal Sci 48: 233-242.

Bashir KM, Choi JS (2017) Clinical and physiological perspectives of β-glucans: The past, present, and future. Int J Mol Sci 18(9): 1906.

Bavei V, Vaezi B, Abdipour M, Kamali MRJ, Roustaii M (2011) Screening of Tolerant Spring Barleys for Terminal Heat Stress: Different Importance of Yield Components in Barleys with Different Row Type. Int. J. Plant Breed Genet. 5 (3): 175-193.

Bhullar S, Jenner C (1985) Differential Responses to High Temperatures of Starch and Nitrogen Accumulation in the Grain of Four Cultivars of Wheat. Funct. Plant Biol. 12: 363–375.

Bustos DV, Riegel R, Calderini DF (2012) Anthocyanin content of grains in purple wheat is affected by grain position, assimilate availability and agronomic management. J. Cereal Sci. 55: 257-264.

Di Silvestro R, Di Loreto A, Bosi S, Bregola V, Marotti I, Benedettelli S, Segura-Carretero A, Dinelli G (2017) Environment and genotype effects on antioxidant properties of organically grown wheat varieties: a 3-year study. J. Sci. Food Agric. 97: 641–649.

EFSA (2011a). Scientific opinion on the substantiation of health claims related to β-glucans from oats and barley and maintenance of normal blood LDL-cholesterol concentrations (ID 1236, 1299), increase in satiety leading to a reduction in energy intake (ID 851, 852), reduction of post-prandial glycaemic responses (ID 821, 824), and "digestive function" (ID 850) pursuant to Article 13(1) of Regulation (EC) No 1924/2006. EFSA Journal, 2011,9 (6), 2207. Available: http://www.efsa.europa.eu/en/efsajournal/pub/2207 [5 June 2020].

EFSA (2011b). Scientific Opinion on the substantiation of health claims related to arabinoxylan produced from wheat endosperm and reduction of post-prandial glycaemic responses (ID 830) pursuant to Article 13(1) of Regulation (EC) No 1924/2006 Available: https://www.efsa.europa.eu/en/efsajournal/pub/2205 [5 June 2020].

Elía M, Slafer GA, Savin R (2018) Yield and grain weight responses to post-anthesis increases in maximum temperature under field grown wheat as modified by nitrogen supply. Field Crops Res. 221: 228–237.

FAOSTAT (2019). Available: http://www.fao.org/faostat/es/ [6 November 2020].

FDA (2008). Food Labeling: Health Claims; Soluble Dietary Fiber from Certain Foods and Coronary Heart Disease. [Docket No. FDA-2008-P0090]. Available: https://pubmed.ncbi.nlm.nih.gov/12361061/ [5 June 2020].

Henry-Kirk RA, Plunkett B, Hall M, McGhie T, Allan AC, Wargent JJ, Espley RV (2018) Solar UV light regulates flavonoid metabolism in apple (*Malus x domestica*). Plant Cell Environ. 41: 675−688.

Henry RJ (1986) Genetic and environmental variation in the pentosan and β-glucan contents of barley, and their relation to malting quality. J. Cereal Sci. 4: 269-277.

Holtekjølen AK, Kinitz C and Knutsen SH (2006) Flavanol and Bound Phenolic Acid Contents in Different Barley Varieties. J. Agric. Food Chem. 54: 2253-2260.

Huang D, Ou B, Hampsch-Woodill M, Flanagan JA, Prior RL (2002) High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. J. Agric. Food Chem. 50: 4437–44.

Iqbal M, Raja NI, Yasmeen F, Hussain M, Ejaz M, Shah MA (2017) Impacts of Heat Stress on Wheat: A Critical Review. Adv. Crop Sci. Tech. 5: 251.

Izydorczyk MS, Dexter JE (2008) Barley. β-glucans and arabinoxylans: Molecular structure, physicochemical properties, and uses in food products - a Review. Food Res. Int. 41: 850-868.

Jenner CF, Ugalde DT, Aspinall D (1991) The physiology of starch and protein deposition in the endosperm of wheat. Aust. J. Plant Physiol. 18:211-226.

Josuttis M, Dietrich H, Treutter D, Will F, Linnemannstöns L, Krüger E (2010) Solar UVB response of bioactives in strawberry (*Fragaria × ananassa Duch*. L.): A comparison of protected and open-field cultivation. J. Agric. Food Chem. 58: 12692−12702.

Kim M-J, Hyun J-N, Kim J-A, Park J-C, Kim M-Y, Kim J-G, Lee S-J, Chun S-C, Chung I-M (2007) Relationship between Phenolic Compounds, Anthocyanins Content and Antioxidant Activity in Colored Barley Germplasm. J. Agric. Food Chem. 55: 4802-4809.

Lancaster JE, Reay PF, Norris J, Butler RC (2000) Induction of flavonoids and phenolic acids in apple by UV-B and temperature. J. Hortic. Sci. Biotech. 75: (2) 142-148.

Li J, Lin X, Chen A, Peterson T, Ma K, Bertzky M, Ciais P, Kapos V, Peng C, Poulter B (2013) Global Priority Conservation Areas in the Face of 21st Century Climate Change. PLoS ONE 8(1): e54839.

Martínez-Lüscher J, Lee Chen CC, Brillante L, Kurtural SK (2017) Partial Solar Radiation Exclusion with Color Shade Nets Reduces the Degradation of Organic Acids and Flavonoids of Grape Berry (*Vitis vinifera* L.) J. Agric. Food Chem. 65: 10693−10702.

Martínez M, Motilva MJ, López de las Hazas MC, Romero MP, Vaculova K, Ludwig IA (2018) Phytochemical composition and β-glucan content of barley genotypes from two different geographic origins for human health food production. Food Chem. 245: 61–70.

Narwal S, Kumar D, Verma RPS (2016) Effect of Genotype, Environment and Malting on the Antioxidant Activity and Phenolic Content of Indian Barley. J. Food Biochem. 40: 91–99.

Pandey KB, Rizvi SI (2009) Plant polyphenols as dietary antioxidants in human health and disease. Oxid. Med. Cell Longev. 2: 270-278.

Passarella VS, Savin R and Slafer GA (2002) Grain weight and malting quality in barley as affected by brief periods of increased spike temperature under field conditions. Aust. J. Agric. Res. 53: 1–9.

Passioura JB (2020) Translational research in agriculture. Can we do it better? Crop and Pasture Sci. 71 (6): 517-528.

Rakszegi M, Lovegrove A, Balla K, Láng L, Bedo Z, Veisz O and Shewry PR (2014) Effect of heat and drought stress on the structure and composition of arabinoxylan and β-glucan in wheat grain. Carbohydr. Polym. 102: 557–565.

Rani S, Chaudhary A, Rani K (2018) Management strategies for abiotic stresses in barley. Wheat and Barley Res. 10(3): 151-165.

Rao S, Santhakumar AB, Chinkwo KA, Blanchard CL (2020) Investigation of phenolic compounds with antioxidant activity in barley and oats affected by variation in growing location. Cereal Chem. 97: 772–782.

Savin R, Molina-Cano JL (2002) Changes in malting quality and its determinants in response to abiotic stresses. In: GA Slafer, JL Molina-Cano, R Savin, JL Araus and I Romagosa (Ed.), Barley Science. Recent advances from molecular biology to agronomy of yield and quality. Food Product Press, New York, USA, pp.523-550.

Savin R, Nicolas ME (1996) Effects of short periods of drought and high temperature on grain growth and starch accumulation of two malting barley cultivars. Aust. J. Plant Physiology 23: 201–210.

Savin R, Stone PJ, Nicolas ME (1996) Responses of grain growth and malting quality of barley to short periods of high temperature in field studies using portable chambers. Aust. J. Agric. Res. 47: 465-477.

Serra A, Rubió L, Macià A, Valls RM, Catalán Ú, de la Torre R, Motilva MJ (2013) Application of dried spot cards as a rapid sample treatment method for determining hydroxytyrosol metabolites in human urine samples. Comparison with microelution solid-phase extraction. Anal. Bioanal. Chem. 405: 9179–9192.

Servei Meteorologic de Catalunya (2017). Available: https://static-m.meteo.cat/wordpressweb/wp-content/uploads/2018/11/22160838/Butllet%C3%AD_primavera_2017_v4.pdf [08 July 2020].

Servei Meteorologic de Catalunya (2018). Available: https://static-m.meteo.cat/wordpressweb/wp-content/uploads/2018/11/22161004/Butllet%C3%AD-primavera-2018_v2.pdf [08 July 2020].

Shamloo M, Babawale EA, Furtado A, Henry RJ, Eck PK, Peter JH, Jones PJH (2017) Effects of genotype and temperature on accumulation of plant secondary metabolites in Canadian and Australian wheat grown under controlled environments. Sci. Rep. 7: 9133.

Sharma A, Shahzad B, Rehman A, Bhardwaj R, Landi M, Zheng B (2019) Response of Phenylpropanoid Pathway and the Role of Polyphenols in Plants under Abiotic Stress. Molecules 24: 2452.

Sinclair TR, Shiraiwa T, Hammer GL (1992) Variation in crop radiation-use efficiency with increased diffuse radiation. Crop Sci. 32: 1281-1284.

Soar CJ, Collins MJ, Sadras VO (2009) Irrigated Shiraz vines (*Vitis vinifera*) up regulate gas exchange and maintain berry growth in response to short spells of high maximum temperature in the field. Funct. Plant Biol. 36: 801-814.

Song RD, Kelman DKL, Johns KL, Wright AD (2012) Correlation between leaf age, shade levels, and characteristic beneficial natural constituents of tea (*Camellia sinensis*) grown in Hawai. Food Chem. 133: 707–714.

Suriano S, Savino M, Codianni P, Iannucci A, Caternolo G, Russo M, Pecchioni N, Troccoli A (2019) Anthocyanin profile and antioxidant capacity in coloured barley. Int. J. Food Sci. Tech. 54: 2478–2486.

Swanston JS, Ellis RP, Perez-Vendrell A, Voltas J and Molina-Cano JL (1997) Pat-terns of barley grain development in Spain and Scotland and their implications for malting quality. Cereal Chem. 74: 456–461.

Tsormpatsidis E, Henbest RGC, Davis FJ, Battey NH, Hadley P, Wagstaffe A (2008) UV irradiance as a major influence on growth, development and secondary products of commercial importance in Lollo Rosso lettuce 'Revolution' grown under polyethylene films. Environ Exp Bot 63: 232–239.

Wallwork MAB, Logue SJ, MacLeod LC, Jenner CF (1998) Effect of high temperature during grain filling on starch synthesis in the developing barley grain. Aust. J. Plant Physiol. 25:173-181.

Wang F, Xu Z, Fan X, Zhou Q, Cao J, Ji G, Jing S, Feng B and Wang T (2019) Transcriptome Analysis Reveals Complex Molecular Mechanisms Underlying UV Tolerance of Wheat (*Triticum aestivum*, L.) J. Agric. Food Chem. 58: 12692–12702.

Yao L, Caffin N, D'arcy B, Jiang Y, Shi J, Singanusong R, Liu X, Datta N, Kakuda Y and Xu Y (2005) Seasonal Variations of Phenolic Compounds in Australia-Grown Tea (*Camellia sinensis*). J. Agric. Food Chem. 53: 6477-6483.

Zadoks JC (1974) Chang TT and Konzak CF, A decimal code for the growth stages of cereals. Weed Res. 14: 415–421.

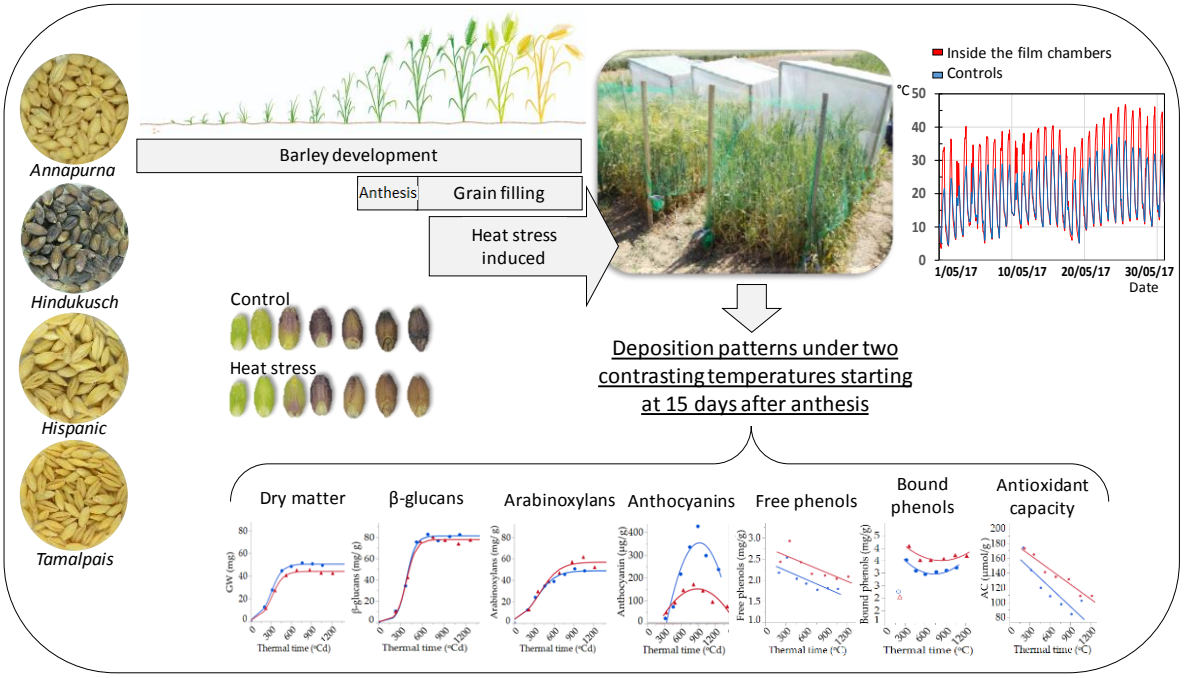
Zhang X, Wu F, Zhang G (2013) Genotypic and Environmental Variations of Arabinoxylan Content and Endoxylanase Activity in Barley Grains. J. Integr. Agric 12(8): 1489-1494.

Zhou B, Jin Z, Schwarz P, Li Y (2020) Impact of Genotype, Environment, and Malting Conditions on the Antioxidant Activity and Phenolic Content in US Malting Barley. Fermentation 6:48.

Chapter III

Impact of Rising Temperature in the Deposition Patterns of Bioactive Compounds in Field Grown Food Barley Grains*

Mariona Martínez-Subirà, Marian Moralejo, Eva Puig, María-iPaz Romero, Roxana Savin and Ignacio Romagosa



Graphical abstract

* Martínez-Subirà, M.; Moralejo, M.; Puig, E.; Romero, M.P.; Savin, R.; Romagosa. Impact of Rising Temperature in the Deposition Patterns of Bioactive Compounds in Field Grown Food Barley Grains. Plants. 2021, 10(3), 598; https://doi.org/10.3390/plants10030598

plants

MDPI

Article

Impact of Rising Temperature in the Deposition Patterns of Bioactive Compounds in Field Grown Food Barley Grains

Mariona Martínez-Subirà, Marian Moralejo, Eva Puig, María-Paz Romero, Roxana Savin and Ignacio Romagosa*

AGROTECNIO-CERCA Center, University of Lleida, Av. Rovira Roure 191, 25198 Lleida, Spain; mariona.martinez@udl.cat (M.M.-S.); marian.moralejo@udl.cat (M.M.); eva.puig@udl.cat (E.P.); mariapaz.romero@udl.cat (M.-P.R.); roxana.savin@udl.cat (R.S.) * Correspondence: ignacio.romagosa@udl.cat; Tel.: +34-973-702-534

Abstract: High temperatures at the end of the season are frequent under Mediterranean conditions, affecting final grain quality. This study determined the deposition patterns throughout grain filling of dry matter, dietary fiber, phenolic compounds and antioxidant capacity for four barley genotypes under two contrasting temperatures. Deposition pattern for dietary fiber followed that of grain weight. Genotypic differences for duration were more significant than for rate. Anthocyanins followed a second-degree polynomial pattern, reaching a maximum before grain maturation. Free and bound phenols decreased as grain developed, suggesting that they are synthesized in early stages. Rate of bound phenols deposition was more sensitive to genotypic changes. Overall, antioxidant capacity decreased over time; the decay being less steep under stress for all genotypes. Heat stress negatively affected grain weight. It did not alter the profile of β-glucans and arabinoxylans deposition but positively changed the accumulation of some phenolic compounds, increasing the antioxidant capacity differentially across genotypes. These results support the growing of food barley in high-temperature stress-prone areas, as some bioactive compound and antioxidant capacity will increase, regardless of the smaller grain size. Moreover, if a market develops for food-barley ingredients, early harvesting of non-mature grain to maximize antioxidant capacity should be considered.

Keywords: *Hordeum vulgare*; grain filling; heat stress; β-glucans; arabinoxylans; phenolic compounds; antioxidant capacity

Citation: Martínez-Subirà, M.; Moralejo, M.; Puig, E.; Romero, M.-P.; Savin, R.; Romagosa, I. Impact of Rising Temperature in the Deposition Patterns of Bioactive Compounds in Field Grown Food Barley Grains. Plants 2021, 10, 598. https://doi.org/10.3390/plants10030598

Academic Editor: Iker Aranjuelo

Received: 26 February 2021
Accepted: 19 March 2021
Published: 22 March 2021

III.1. Introduction

Barley (*Hordeum vulgare* L.) grain contains a variable amount of bioactive compounds with known health-promoting properties, such as dietary fiber (β-glucans, arabinoxylans, cellulose, lignin, and lignans), phenolic compounds, tocols, sterols and folates (Idehen et al., 2017). β-glucans are major non-starch polysaccharides present in cell walls. They are linked to the maintenance of normal blood cholesterol levels (EFSA, 2011) and the reduction of blood glucose after meals (FDA, 2008), as well as improving the responsiveness of the immune system to infectious diseases, inflammation and some types of cancer (De Graaff et al., 2018). Arabinoxylans constitute a fraction of dietary fiber, which have positive effects on the human digestive system (Biliaderis and Lazaridou, 2009). Phenolic compounds are a large class of secondary plant metabolites, which can be found free or bound to compounds in the cell wall of the barley grain. Phenolic compounds, with their strong antioxidant power, are associated with the reduction of cardiovascular disease, inflammation and a diversity of cancers (Pandey and Rizvi, 2009). Interest in the health benefits of barley has led to an increased focus on these bioactive compounds in mature grain (Suriano et al., 2018; Martínez et al., 2018).

The effective grain filling phase (between the end of the lag phase and physiological maturity) is when dry matter and bioactive compounds accumulate, determining the final weight and nutrient composition and the quality of the grain (Schulman, 2000). It is well documented that the accumulation of dry matter during grain filling follows a sigmoid pattern (Dodig et al., 2018). This is characterized by three phases: lag phase (fertilization and rapid cell division), the effective grain filling period (accumulation of reserve components) and the maturation drying phase (loss of water content and reaching "physiological maturity", i.e., maximum dry matter accumulation) (Loss et al., 1989; Egli, 2006). In contrast, less is known about the accumulation patterns of the bioactive compounds during barley grain filling. Changes in non-starch polysaccharide accumulation during cereal grain development have been observed. It has been reported that β-glucans increase linearly, starting during endosperm development and continuing until ripening in barley grain (De Arcangelis et al., 2019). In wheat, which is often used as a model similar to barley, the β-glucan concentration initially increases and then decreases slowly throughout development to a low concentration at maturity (Verspreet et al., 2013). Arabinoxylan was reported to appear in barley during early cellularization, changing its structures during endosperm development from a highly substituted form to a less substituted form (Wilson et al., 2012). Similar to barley, the distribution pattern of arabinoxylans deposition in wheat shows a rapid increase in concentration at the end of the cell division and expansion phase, until it reaches the maturation phase where it remains constant (Verspreet et al., 2013). Different patterns of variation in phenolic compound content have also been observed during grain filling. For instance, in wheat, total bound phenolics peak in the early stage of development (Ma et al., 2016), while the anthocyanin concentration first increases sharply during the immature phase and then decreases from 25-33 days after anthesis and onward (Knievel et al., 2009).

The Intergovernmental Panel on Climate Change (IPCC) has projected that the global warming trend from 1986-2005 to 2081-2100 will show a temperature increase of 0.3 °C to 1.7 °C (IPCC, 2013). Therefore, current concerns exist on the impact of global climate change on the production of crops such as barley (Ko et al., 2019; Ezquer et al., 2020; Dell'Aversana et al.,2021). Future growing conditions will expose plants to variable and extreme climate change factors, impacting global agriculture, so future research in this area is essential (Ezquer et al., 2020) to take adequate adaptation measures. High maximum temperature during the grain filling period is one of the most

relevant abiotic stresses under Mediterranean conditions. In fact, it is expected to be more frequent due to climate change (Li et al., 2013). The clear effects of high temperature on the reduction of grain weight are well documented in the literature (Savin and Nicolas, 1996). However, high temperatures can also induce various physiological, biochemical and molecular responses in plants. In a recent study, we observed that the thermal stress during grain filling affects the final grain weight and changes the relative composition of β-glucans, arabinoxylans and more than 50 phenolic compounds in the mature grain (Martínez-Subirà et al., 2021). Therefore, understanding the dynamics of the accumulation of these bioactive compounds under heat stress is another crucial aspect for enhancing the nutritional value of the barley grain at harvest. Insights into the time course and compositional changes of bioactive compounds during grain development is an important aspect for improving the nutritional quality of barley. Hence, in the present study, we compared the pattern of accumulation of dry matter and bioactive compounds (β-glucans, arabinoxylans, total free and bound phenolic compounds and their antioxidant capacity) in four barley genotypes exposed to continuous high temperature under field conditions. Identifying the timeframe when the maximum content of these bioactive compounds may occur could be useful for agronomic practices and also for promoting further research into whether non-mature barley grain could be used as a functional ingredient in the elaboration of healthy cereal-based food products. Therefore, the main aims of this study were (i) to determine the deposition pattern and antioxidant capacity throughout grain filling and (ii) to analyze the effect of high temperature on the accumulation of these components. This research could provide further knowledge about the deposition patterns of different bioactive compounds in barley under two contrasting temperatures in field conditions since the few published works in barley have focused on changes in deposition patterns of individual bioactive compounds under stress-free conditions (Wilson et al., 2012; De Arcangelis et al., 2019). To the best of our knowledge, this is the first time that a study has explored the deposition patterns of the main health-promoting components in food barley genotypes growing in field conditions under two contrasting temperatures

III.2. Results

III.2.1. Grain Weight

As expected, the standard 3-parameter logistic growth was found to be the most appropriate model for describing the grain filling process for grain weight (GW) in the 16 genotypes x year x environment combinations (Figure III.1A). The overall R2 value for the fitting of the 16 logistic curves to the GW data was ca. 99%. Table III.1 shows the partitioning of variability for maximum grain weight, growth rate and duration for the 16 standard logistic curves in Figure III.1A.

A significant environmental effect was detected for maximum grain weight, which was quantitatively more important than the genotypic effects and the other terms in the model. In the control treatment, average maximum grain weight was highest for Hispanic (54.0 ± 6.4 mg), followed by Annapurna (51.7 ± 1.2 mg), Hindukusch (44.2 ± 0.3 mg) and Tamalpais (43.1 ± 1.6 mg) (Figure III.1A). The maximum grain weight of the heat stressed plants were lower than in the control plants (7-22% average decrease). Although not significant, the reduction in maximum grain weight caused by high temperature seemed to be more related to the rate ($p = 0.1207$) than the duration ($p = 0.9669$) of the grain filling and a small, non-significant reduction in the grain filling rate was most often observed under heat stress. Significant variations in the duration of grain weight among genotypes were observed during grain filling (Table III.1).

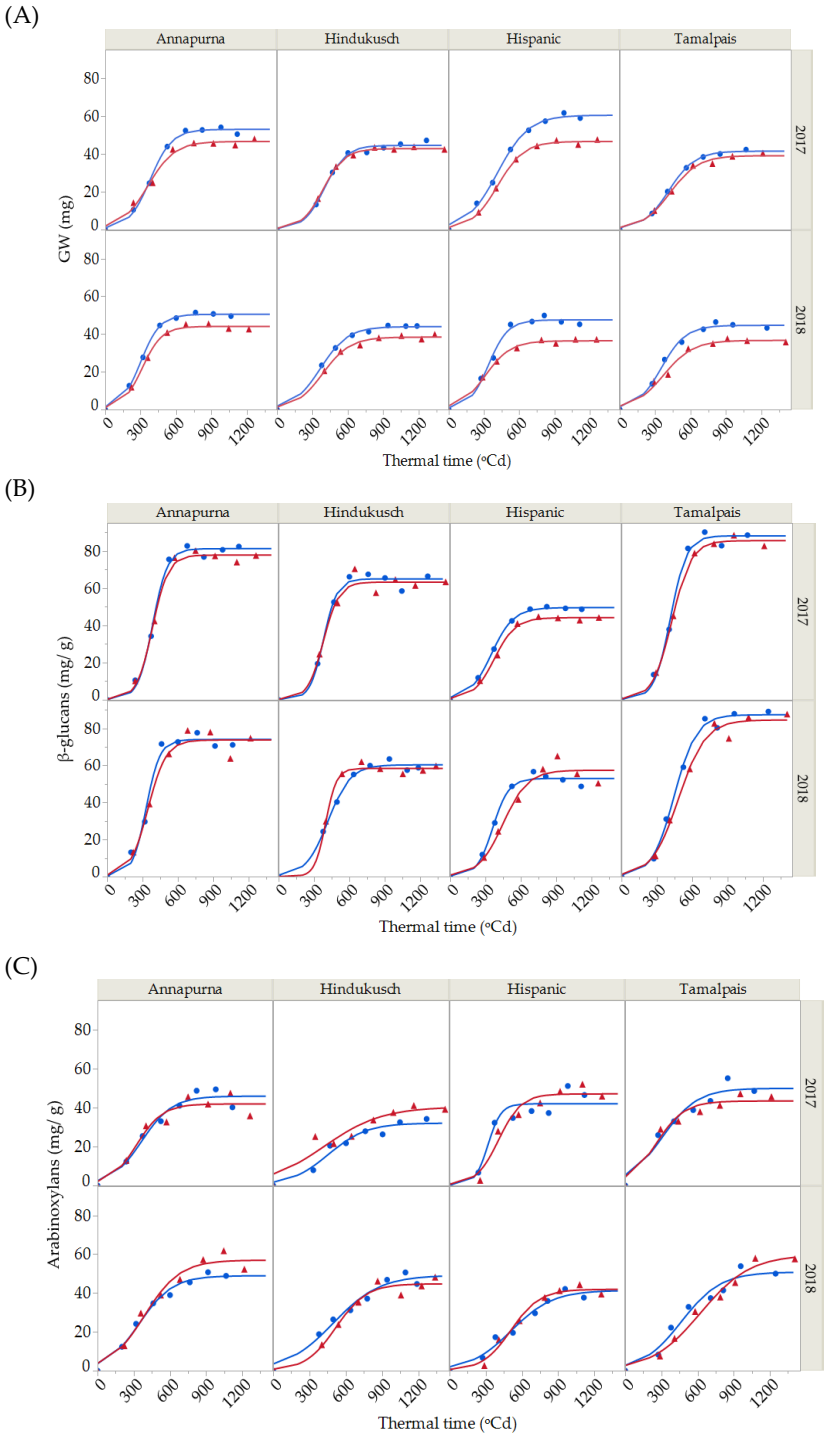


Figure III.1. (A) Dynamics of grain weight growth, **(B)** β-glucans and **(C)** arabinoxylan contents for the two seasons (2017 and 2018) and four barley genotypes under control (blue points) and thermal stress conditions (red triangles). Solid lines represent the 3-parameter logistic fit for control (blue) and stressed conditions (red) for grain weight, β-glucans and arabinoxylan (Total R^2 = 99.07%, R^2 = 98.30% and R^2 = 94.54%, respectively).

On average, the duration of the period was shorter for Annapurna compared with the other genotypes. A lack of statistical significance for the genotype x environment interaction for any of the three parameters (growth rate, duration and maximum) (Table III.1) was surprising, as we could

infer from Figure III.1 that some genotypes seemed to be more affected by high temperatures than others, i.e., Hispanic *vs*. Hindukusch. This could be related to the model structure in which interactions involving the year were pooled into the error term. This was a consequence of the absence of full replications, resulting in poor detection power.

Table III.1. Analyses of Variance for the Estimates of the Grain Weight (mg) Deposition Parameters: Maximum Weight, Growth Rate and Duration.

		Maximum Weight			**Growth Rate**			**Duration**		
Source	df	Sum of Squares	*F* Ratio	*p*-value	Sum of Squares	*F* Ratio	*p*-value	Sum of Squares	*F* Ratio	*p*-value
Corrected total	15	582.0			0.000028960			87,893		
Year	1	64.1	4.78	*0.0651*	0.000000004	0.00	*0.9887*	44,123	28.62	***0.0011***
Genotype: G	3	188.0	4.67	***0.0427***	0.000001001	1.74	*0.2579*	25,485	4.95	***0.0376***
Environment: E	1	191.3	14.26	***0.0069***	0.000006260	3.27	*0.1207*	3	0.00	*0.9699*
G x E	3	44.3	1.11	*0.4061*	0.000003110	0.54	*0.6745*	1286	0.25	*0.8591*
Residual	7	93.9			0.000011500			12,016		

A bold number indicates statistical significance at $\alpha < 0.05$.

III.2.2. β-Glucan and Arabinoxylan Contents

β-glucan concentration steadily rose at the beginning of the grain filling period and then accumulated substantially between the 600 to 800 growing degree-days after anthesis (GDA; Figure III.1B), reaching a maximum peak. Thereafter, the β-glucan content did not change substantially. The maximum, rate growth and duration for β-glucans during grain filling were estimated from fitting the logistic curve and analyzing the variance for the estimates of β-glucans, as shown in Table III.2.

Table III.2. Analyses of Variance for the Estimates of β-glucan Content (mg/g) Deposition Parameters: Maximum Content, Growth Rate and Duration.

		Maximum Content			**Growth Rate**			**Duration**		
Source	df	Sum of Squares	*F* Ratio	*p*-value	Sum of Squares	*F* Ratio	*p*-value	Sum of Squares	*F* Ratio	*p*-value
Corrected total	15	3112.0			0.00017606			94,632		
Year	1	2.2	0.10	*0.7571*	0.00000210	0.22	*0.6573*	4340	1.22	*0.3065*
Genotype: G	3	2946.9	46.05	***0.0001***	0.00007037	2.39	*0.1543*	51,292	4.79	***0.0403***
Environment: E	1	11.8	0.55	*0.4830*	0.00000166	0.17	*0.6933*	5412	1.52	*0.2578*
G x E	3	2.4	0.04	*0.9885*	0.00003325	1.13	*0.4003*	8617	0.81	*0.5297*
Residual	7	148.7			0.00006867			24,971		

A bold number indicates statistical significance at $\alpha < 0.05$.

In this study, the only significant effect associated with the accumulation of β-glucans was the genotype. Tamalpais and Annapurna, known as high β-glucan content genotypes, had maximum β-glucan concentration values of 87.8 ± 0.4 mg/g and 77.6 ± 3.6 mg/g, respectively, followed by Hindukusch (62.7 ± 2.3 mg/g) and Hispanic (51.3 ± 1.7 mg/g). Genotypic variations were also found for the duration of β-glucan accumulation. Again, on average, Annapurna needed less thermal time to increase the content of β-glucans from 5% to 95% of the final value during grain filling. No significant environment or genotype x environment interaction was detected (Table III.2).

During grain filling, the arabinoxylan content also increased rapidly from shortly after the end of anthesis (200 GDA) up to the maturation and desiccation phase (800 GDA) (Figure III.1C). Arabinoxylans were fit by a logistic curve with an overall R2 of 94.54%. Partitioning of the total variability of arabinoxylans is shown in Table III.3, with the maximum content, growth rate and duration for the 16 logistic curves shown in Figure III.1C. Neither the environment nor the main genotype main effects showed statistical differences for any of the three parameters. The year that is, the uncontrolled environmental differences associated with the growing season was the only significant main effect and only for duration.

Table III.3. Analyses of Variance for the Estimates of Arabinoxylan Content (mg/g) Deposition Parameters: Maximum Content, Growth Rate and Duration.

		Maximum Content			**Growth Rate**			**Duration**		
Source	df	Sum of Squares	*F* Ratio	*p*-value	Sum of Squares	*F* Ratio	*p*-value	Sum of Squares	*F* Ratio	*p*-value
Corrected total	15	674.1			0.00016401			52,3370		
Year	1	165.8	4.51	*0.0714*	0.00002439	2.25	*0.1775*	22,7143	9.03	***0.0198***
Genotype: G	3	233.3	2.11	*0.1870*	0.00005678	1.75	*0.2436*	10,2788	1.36	*0.3304*
Environment: E	1	16.0	0.43	*0.5310*	0.00000077	0.07	*0.7973*	7346	1.29	*0.6057*
G x E	3	1.3	0.01	*0.9981*	0.00000652	0.20	*0.8925*	9969	0.13	*0.9379*
Residual	7	257.6			0.00007566			17,6124		

A bold number indicates statistical significance at $\alpha < 0.05$.

As shown in Tables III.1, III.2 and III.3, durations of grain weight and fiber deposition were, in general, more affected by year than by genotype or by the imposed thermal stress. Figure III.2 shows the average duration for these three variables across years and control *vs.* stress conditions for the four genotypes. No differences in the duration of the three variables were found in the first year ($p = 0.1908$, ANOVA table not shown). However, significant differences were found among the three variables for the second season ($p = 0.0001$, ANOVA table not shown). In 2018, a more favorable year in terms of meteorological conditions during grain filling, deposition of arabinoxylans took longer than for GW and β-glucans under stress and control conditions and for the four genotypes.

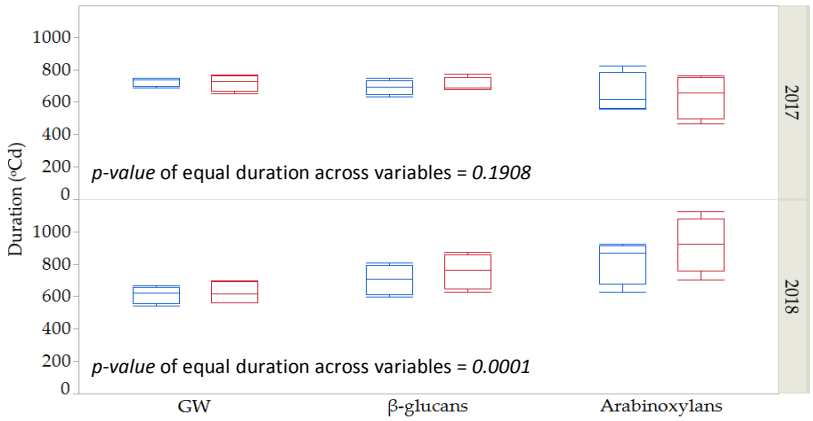


Figure III.2. Estimates and standard error for duration for grain weight (GW), β-glucan and arabinoxylan contents across years (2017 and 2018) under control (blue) and stressed conditions (red) among four genotypes.

III.2.3. Anthocyanin Contents

Although anthocyanins were recorded during the course of grain filling for all four genotypes (Figure III.3) in this study, the dynamics of anthocyanin deposition were only studied in Hindukusch, the only purple genotype, as the other three non-colored genotypes showed irrelevant extremely low values. The anthocyanin content in Hindukusch during the course of grain filling were best described by a second-degree curve (Figure III.3). The synthesis of anthocyanins started relatively late in grain filling (at about 400 GDA), reaching a maximum peak in maturity (800 GDA) and decreasing throughout grain desiccation. The average maximum content during both growing seasons was 383 ± 49 µg Cy-3-glu/g in the control and 226 ± 85 µg Cy-3-glu/g in the heat treatment. Thus, the reduction of anthocyanin content between the maximum values reached at maturity until harvest time was in the order of 40% (Figure III.3).

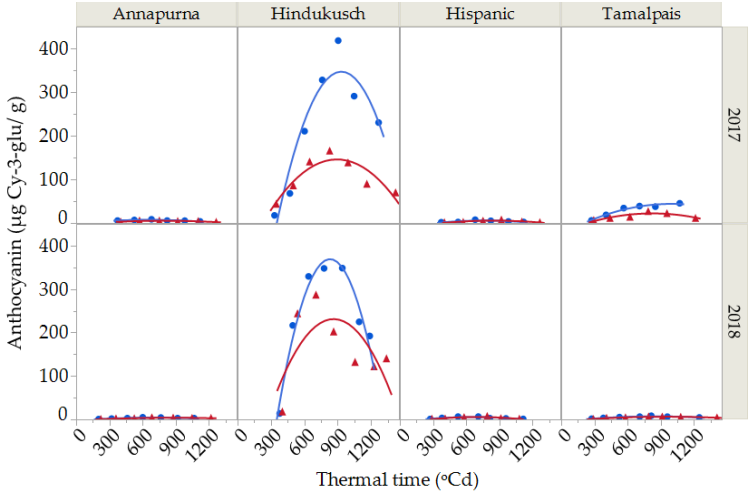


Figure III.3. Anthocyanin content during grain filling for two seasons (2017 and 2018) and for the purple barley genotype (Hindukusch) under control conditions (blue circles) and thermal stress (red triangles). Solid lines represent the best fit (second-degree polynomial curve, Total R2 = 86.45%) for control (blue) and stressed (red) conditions, respectively.

The anthocyanin content of barley grain grown under plastic cover was significantly lower than in the controls (Figure III.3, Table III.4). Furthermore, the significant environment x thermal time interaction suggested that the rate of deposition of anthocyanins changes under control and stress conditions (Table III.4).

Table III.4. Analysis of variance for anthocyanin content (µg Cy-3-glu/g) according to a second-degree curve for thermal time.

Source	df	Sum of Squares	*F* Ratio	*p*-value
Corrected total	27	34,3845		
Thermal time: TT	2	18,7749	32.24	***0.0000***
Year: Y	1	3215	1.10	*0.3090*
Environment: E	1	49,574	17.03	***0.0008***
Y x E	1	3111	1.07	*0.3166*
TT x Y	2	12,908	2.22	*0.1413*
TT x E	2	37,208	6.39	***0.0091***
TT x Y x E	2	3498	0.60	*0.5603*
Residual	16	46,581		

A bold number indicates statistical significance at $\alpha < 0.05$.

III.2.4. Free and Bound Phenol Contents

Free and bound phenols were determined for the four genotypes under normal and stress conditions in the course of grain filling in the second year. Free phenols continuously decreased throughout the grain filling period (Figure III.4A) by around 30% between the maximum content at the beginning of grain filling until harvest time. Their dynamics were best fitted by a simple first-degree linear model, which was used for partitioning total variability (Table III.5). The bound phenol content was more variable, fluctuating from one genotype to another. The bound phenols content decreased for Hispanic and Tamalpais with time (Figure III.4B). However, Hindukusch and Annapurna followed a different pattern. Initially, the bound phenolic decrease occurred similarly to the other genotypes, but the drop rate reduced after 700 GDA and remained relatively constant or increased significantly in the case of Hindukusch. High temperature exposure did not alter these patterns of accumulation of bound phenols in all genotypes.

Table III.5. Analyses of variance of total free and bound phenolic compound contents (mg Gallic acid equivalent (GAE)/g) and antioxidant capacity (µmol Trolox/g) according to a first- or second-degree curve with thermal time.

Source	**Free Phenols (First-Degree Model)**				**Bound Phenols (Second-Degree Model)**				**Antioxidant Capacity (First-Degree-Model)**			
	df	Sum of Squares	*F* Ratio	*p*-value	df	Sum of Squares	*F* Ratio	*p*-value	df	Sum of Squares	*F* Ratio	*p*-value
Corrected total	55	7.751			47	11.95			55	46,131		
Thermal time: TT	1	3.364	92.31	***0.0000***	2	0.75	22.78	***0.0000***	1	29,490	390.43	***0.0001***
Genotype: G	3	1.392	12.73	***0.0000***	3	6.44	129.70	***0.0000***	3	15,590	68.80	***0.0001***
Environment: E	1	0.409	11.22	***0.0027***	1	2.80	169.11	***0.0000***	1	3274	43.34	***0.0001***
G x E	3	0.793	7.25	***0.0013***	3	0.08	1.66	*0.2030*	3	1073	4.73	***0.0064***
TT x G	3	0.142	1.30	*0.2981*	6	1.42	14.26	***0.0000***	3	125	0.55	*0.6495*
TT x E	1	0.127	3.48	*0.0743*	2	0.01	0.33	*0.7206*	1	909	12.03	***0.0013***
TT x G x E	3	0.068	0.62	*0.6088*	6	0.06	0.61	*0.7226*	3	190	0.84	*0.4801*
Residual	40	1.458			24	0.40			40	3021		

A bold number indicates statistical significance at $\alpha < 0.05$.

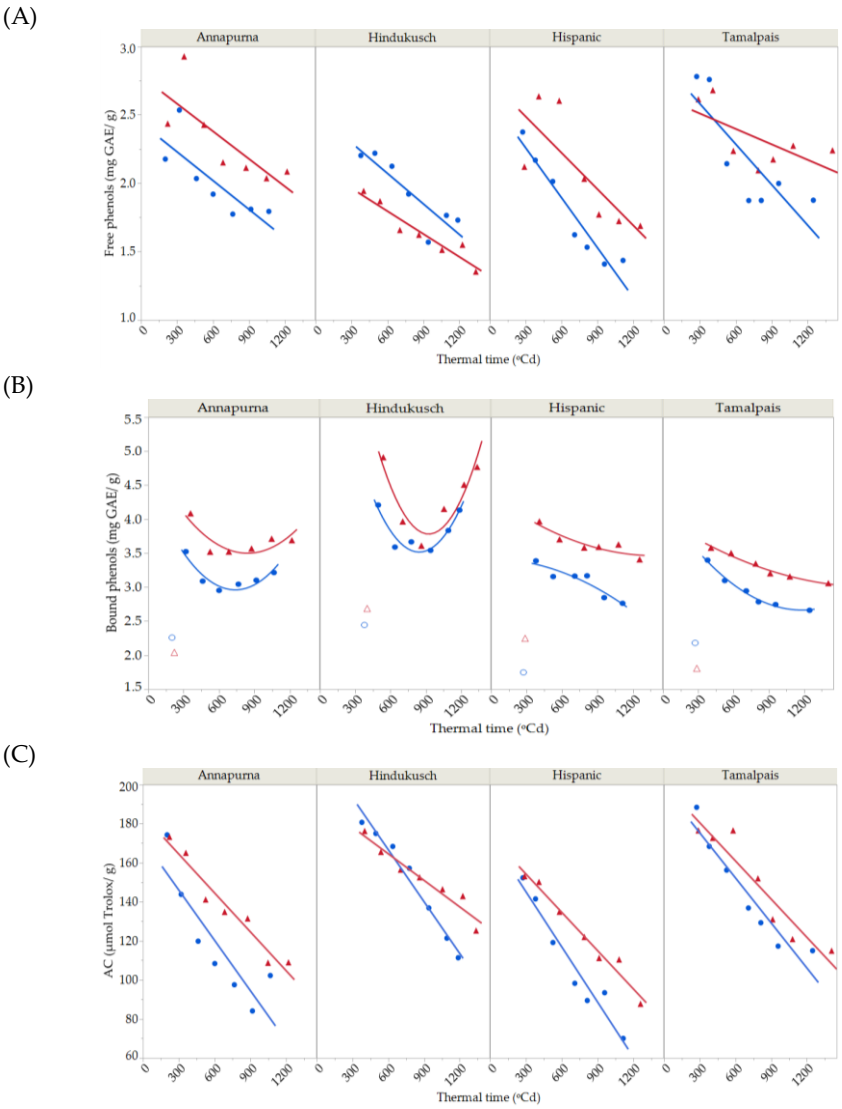


Figure III.4. Dynamics of **(A)** free and **(B)** bound phenolic compound content and **(C)** antioxidant capacity during grain filling for four barley genotypes in 2018 under control (blue circles) and thermal stress conditions (red triangles). Solid lines represent the best fit (first- and second-degree polynomial curve for free and bound phenolic compounds and first-degree for antioxidant capacity. Total R2 = 81.19% and R2 = 96.65%, R2 = 93.45%, respectively).

Both genotype and environment—that is, control *vs*. imposed heat stress, main effects and their interaction—were significant for free phenols (Table III.5). Overall, the genotype seemed to be more important than the environment. Under heat stress, concentrations were higher for the three non-colored genotypes (7-16% average increase during grain filling) but not for Hindukusch, which had more free phenols in the control. The lack of significant interactions with thermal time suggested a common dynamic of free phenol content across genotypes, control *vs.* imposed stress. Bound phenol concentrations were significantly affected by genotype and environment main effects, but no genotype x environment interaction was detected. The significant genotype x thermal time

interaction for bound phenols suggested that the rate of deposition of these compounds changes with genotype, as seen in Figure III.4B. Hindukusch clearly behaves differently than the others.

In the control treatment, the maximum content of free phenols in immature grain was observed in Tamalpais (2.8 ± 0.1 mg GAE/g), while the highest levels of bound phenols were seen in Hindukusch (4.2 ± 0.1 mg GAE/g). These two genotypes maintained their higher contents compared to the other genotypes once the grain was ripe, but the free phenol content decreased by approximately 30% for Tamalpais and the bound phenol content decreased by approximately 3% for Hindukusch at the end of the grain filling.

III.2.5. Antioxidant Capacity

Antioxidant capacity sharply decreased as dry matter increased during grain filling (Figure III.4C). The antioxidant capacity ranged from 152 ± 7 to 188 ± 9 µmol Trolox/g in the first measurements and from 70 ± 1 to 115 ± 3 µmol Trolox/g at the end of the experiment, a decrease of around 40% during grain growth. The antioxidant capacity was modelled by a simple first-degree model, which explained 95.36% of the total variability. Partitioning of the total variability for antioxidant capacity based on this linear trend is shown in Table III.5. Genotype, environment and its interaction were highly significant. Tamalpais and Hindukusch had the highest antioxidant capacity values both in early immature grain (188 ± 9 and 181 ± 5 µmol Trolox/g) and mature grain (115 ± 3 and 111 ± 2 µmol Trolox/g). This was expected as these two genotypes had the highest content of free and bound phenols. The antioxidant capacity was higher for genotypes grown under heat stress (13%), except for Hindukusch. The highest increase in the antioxidant capacity due to heat stress was observed in Annapurna and Hispanic (on average 26% and 13%, respectively), the two genotypes that also had a greater increase in free (16% and 18%, respectively) and bound phenols (16% and 12%, respectively) due to the imposition of thermal stress. The thermal time x environment interaction was also significant, suggesting that the decay observed in the control plots was steeper than for the induced-stressed ones (Figure III.4C).

III.3. Discussion

Barley (*Hordeum vulgare* L.) grains are rich in bioactive compounds with health-promoting properties, which are genetically and environmentally regulated. The four different barley genotypes selected for this study widely differed in an array of bioactive compounds, potentially susceptible to heat stress from 15 days after heading to physiological maturity.

As discussed in previous studies (Elía et al., 2018; Martínez-Subirà et al., 2021) the use of polyethylene film chambers to increase the maximum temperature under field conditions resulted in reduced incident radiation (up to 15% at noon on very sunny days). The relationship between direct and diffuse incoming radiation was changed, favoring the latter. Then, the reduction in incoming radiation was compensated by increasing the radiation use efficiency, and the source-balance hardly changed during grain filling (Elía et al., 2018). Therefore, the main effect on grain weight was due to the increase in maximum temperatures.

The duration of grain filling was not affected by heat stress when measured on growing degree-days, as shown by others (Dias and Lidon, 2009). The differences in final grain weight between the controls and heat conditions might be due to the differences in the rate of the grain filling period since a positive correlation was found between growth rate and the effect of high temperatures on the maximum weight. In fact, some authors have suggested the selection of genotypes with higher filling rates as the best strategy for increasing grain weight (Dias and Lidon, 2009). Then, the weight

reduction by thermal stress exposure was probably more related to a direct effect on the grain growth capacity, as proposed by MacLeod and Duffus (1988). High temperatures may induce inactivation of sucrose synthase, reducing the starch synthesis, reflected in the reduction of grain weight.

Barley is an important source of dietary fiber (β-glucans and arabinoxylans). No apparent dilution effect of these carbohydrates was detected with grain growth. The accumulation of β-glucans (expressed in mg/g of dry matter) through grain filling also followed a sigmoid pattern, very close to that of grain weight, suggesting that β-glucans were synthesized all through grain filling at the same rate as dry matter. The arabinoxylan content also followed a sigmoid pattern, although the fit to the 3-parameter logistic model was not as good as that for grain weight and β-glucans. According to De Arcangelis et al. (2019), β-glucans increased linearly from endosperm development to maturation, whereas, seemingly contrary to our findings, Wilson et al. (2012) reported that arabinoxylans accumulated significantly during early cellularization and changed their structures during grain development. They observed that the xylan backbone is heavily substituted with arabinose residues during early grain development. The apparent contradiction between early synthesis and our continuous deposition could be related to the analytical method used to quantify arabinoxylans. The standard D-xylose enzymatic procedure may not be fully adequate to quantify arabinoxylan levels during the early stages of grain filling, where the polysaccharide is in a highly substituted form with arabinose side chains. Future studies would be necessary to determine whether greater quantification of arabinoxylan can be obtained by including a pre-treatment with debranching enzymes such as arabinofuranosidase in the early stages of grain filling.

In this study, genotypic differences were more important than environmental effects on the β-glucan and arabinoxylan contents. Although we expected more significant differences associated with environmental factors, as reported by Swanston et al. (1997), our results suggest that the content of β-glucans in barley was determined mainly by the genotype. This is in line with Molina-Cano et al., (1996) who also considered that the genetic effects were more important than the environmental conditions in the final β-glucan content. Despite the few studies that have examined the variation in arabinoxylan levels due to heat stress, it has been shown that the heat effect increased the total arabinoxylan content in wheat (Rakszegi et al., 2014). However, we have previously observed that the final arabinoxylan content in barley was actually caused by an indirect concentration effect of the same amount of arabinoxylans in lighter grains and not by an apparent direct response to the heat stress imposed (Martínez-Subirà et al., 2021).

In general, no statistical differences were found for the duration of dry weight, β-glucan and arabinoxylan deposition during grain filling, suggesting that the maximum content for dietary fiber roughly coincides with maximum dry weight, that is, at or near physiological maturity. However, in 2018, a more favorable year in term of meteorological conditions during grain filling, the deposition of arabinoxylans took longer than for GW and β-glucans under stress and control conditions and for the four genotypes, suggesting that the thermal time of analyzable arabinoxylan accumulation could be more sensitive to uncontrolled environmental changes.

The anthocyanin content followed a second-degree pattern during the course of grain filling. Anthocyanins were synthesized relatively late in grain filling, reaching a peak at maturity and then decreasing through grain desiccation. This is in line with other findings for wheat (Knievel et al., 2009) and rice (Shao et al., 2013). This could be due to differences in the development rate between

the endosperm and external layers (Zhang et al., 2010). The reduction after the maximum anthocyanin content occurred in the phase of desiccation, that is, when the grain filling period was almost completed and thus was not caused by a biomass dilution effect. On the contrary, Bustos et al. (2012) suggested that the reduction of the anthocyanin content was conditioned by the availability of assimilates in the final phase of grain filling.

The anthocyanin content decreased in the stressed treatment. Imposing high temperatures by covering the field plots with a polyethylene film also reduced the incident radiation and this decrease could be likely the cause for the reduction in the anthocyanin content. The genes that control anthocyanin biosynthesis are positively regulated by light (Ahmed et al., 2009), so the incidence of solar radiation had a direct effect on the accumulation of anthocyanins. Bustos et al. (2012) also observed a decrease in the anthocyanin content in wheat grains from shading the ears. Anthocyanins acted as a specific light protector and their high content favored the absorption and tolerance to ultraviolet radiation, as well as increasing their antioxidant capacity (Sharma et al., 2019). Therefore, blocking UV radiation by conventional polyethylene film (Tsormpatsidis et al., 2008) affected the rate of anthocyanin deposition in the barley grains.

The free phenolic compounds decreased toward grain maturation for all four genotypes in both stress and control conditions, as also reported in wheat during grain development (Özkaya et al., 2018). The bound phenol concentrations did not follow this linear trend, rather they followed a second-degree curve as the grain developed. The bound phenols in Hindukusch, the purple grain genotype, increased sharply toward the end of grain filling. Ma et al. (2016) reported that the highest bound phenol biosynthesis occurs later in the development of purple wheat than in the non-colored genotypes. In a previous study, we observed that the main phenols detected in the bound phenolic fraction in the mature grain were phenolic acids (Martínez-Subirà et al.,2021). These compounds were also detected to a lesser extent in the free phenolic fraction. Furthermore, among these genotypes, Hindukusch had the highest bound phenolic acid content (Martínez-Subirà et al., 2021). Therefore, the bound phenol accumulation could be attributed to conversion between fractions (from free to bound) during grain filling.

The higher levels of phenolic compounds in the early stages of grain filling may be a consequence of an early activity of phenylalanine ammonia lyase (PAL) (McCallum and Walker, 1991) the enzyme that catalyzes the conversion of phenylalanine to trans-cinnamic acid during phenolic compounds biosynthesis (Sharma et al., 2019). The reduction in phenolic compounds during grain filling was mainly due to a dilution effect, as starch was deposited in the growing endosperm (Knievel et al., 2009). Several studies have speculated that sucrose increased PAL activity and induced the production of phenolic compounds during the development of different plant species (Gonzalez-San José and Diez, 1992; Solfanelli et al., 2006; Guo et al., 2011). The carbohydrate availability could have been compromised primarily during starch synthesis in later grain filling stages (Schulman et al., 2000), affecting the phenolic compound biosynthesis during the final grain development. Other authors suggested that the reduction in phenolic contents during grain maturation was more closely related to other physiological processes, such as a decrease in photosynthesis or oxidative metabolism during the grain dehydration process (Özkaya et al., 2018).

Genotypic effects were more important than environmental conditions for free and bound phenolic contents. However, free and bound phenolic contents changed differentially between genotypes with the severity of heat stress. Our results show that the bound phenols were more stable under

high temperature stress while the free phenols showed greater variability to thermal stress, corroborating the results observed by Silvestro et al. (2016). These authors showed that free phenols increased more under harsh climatic conditions than bound phenols. However, this was not observed for the colored genotype under stress. This showed less free phenol content than under the control conditions. The reduction of anthocyanins associated with the polyethylene film covering could explain why the concentrations of free phenols did not increase in this genotype. Heat stress also had a significant impact on the bound phenol content in all genotypes. An increased rate of bound phenol accumulation was sustained throughout the period of high temperatures in all genotypes. Such high temperatures have proven favorable to phenolic synthesis in wheat varieties (Shamloo et al., 2017). Plants synthesized more phenolic compounds under temperature stress, which ultimately protected these plant cells from heat-induced oxidative damage (Sharma et al., 2019). Therefore, the increased accumulation of these compounds was accompanied by enhanced tolerance of the plants to high temperatures. Previous research suggested that high temperature influenced the metabolic pathway of phenolic compounds by augmenting the PAL activity, thus increasing the content of some phenolic compounds, which protected plants against heat stress (McCallum and Walker, 1991; Wu et al., 2016).

The antioxidant properties constantly decreased during grain development, as reported in rice (Shao et al., 2014) and wheat (Özkaya et al.2018). Antioxidant molecules had less activity in the final stage of grain maturation due to the decrease in some physiological processes, such as photosynthesis or oxidative metabolism during the grain dehydration process, as proposed by Özkaya et al. (2018). Reactive oxygen species were generated during these physiological processes. Thus, if these processes were decreased during the grain dehydration (final stage of grain filling), the antioxidant molecules levels generated by the grain decreased consequently. Hence, the antioxidant capacity progressively decreases as the grain matures. Free and bound phenols make a significant contribution to the total antioxidant capacity, as described in the literature (Zhu et al., 2015; Suriano et al., 2018). The increase in the antioxidant capacity due to heat stress was related to the increase in phenolic compounds. This could have occurred to protect the plant cells from heat-induced oxidative damage, as mentioned above.

III.4. Materials and Methods

III.4.1. Field site Description, Treatments and Experimental Conditions

The field experiments were carried out under irrigated and well-fertilized conditions during the 2016-2017 and 2017-2018 growing seasons at Semillas Batlle SA, Bell-Lloc d'Urgell (41°37'N, 0°47E), Northeast Spain. Four food barley genotypes were grown under two temperature conditions after heading. The genotypes were Annapurna, Hindukusch, Hispanic and Tamalpais, parents in our food barley breeding program, and they differed in number of rows, presence/absence of hulls, type of starch, grain quality and color (their grain characteristics appear in Table III.S1). The temperature treatments were: a control and a high temperature treatment starting 15 days after heading (decimal code (Zadoks et al., 1974), DC55) and continuing to physiological maturity (DC 90). The main plot size was 4 m x 1.5 m (six rows separated 20 cm) from which two subplots of the same size were established in order to apply the control and high temperature treatments from mid-April to late June. The seeding rate was 350 seeds/m^2. All plots (control & heat stress) were flood-irrigated twice, once before heading and a second time in the first part of grain filling (60 mm each time), to assure that they did not suffered of any water stress. Biotic interferences were avoided through

controlling weeds, insects and diseases following usual practices. The heat treatment was imposed by covering half of the plot with transparent polyethylene film (125 µm) mounted on wooden structures 1.5 m above the soil level, as described in Elía et al. (2018) but leaving the bottom 30 cm of the four sides of each structure open and the top punctured in order to facilitate free gas exchange. To monitor the air temperature, regularly distributed temperature sensors connected to data loggers were placed inside and outside the structures at the height of the spikes. The structures increased the maximum temperature by up to 8 °C, while the polyethylene film reduced solar radiation by up to 15%. The average daily temperatures in the spring growing period were higher (15 °C *vs.* 13 °C), precipitation lower (100 L/m2 *vs*. 175 L/m^2) and solar radiation more intense (+10% *vs.* -10% long-term average) in 2017 than 2018 (Servei Meteorologic de Catalunya). For more details see Martínez-Subirà et al. (2021)

III.4.2. Grain Weight and Milling

Individual spikes were marked at anthesis to monitor grain growth. At seven-day intervals during grain development, from seven days after anthesis until harvest maturity, ten spikes were collected from each subplot. The spikes (a total of seven samples per sub-plot) were lyophilized and threshed using a HALDRUP LT-15 laboratory thresher (HALDRUP GmbH, Ilshofen, Germany). Once the grains were threshed, thousand grain weight (GW) was determined with a Marvin system (GTA Sensorik GmbH, Neubrandenburg, Germany) according to the standard MSZ 6367/4-86 (1986) method. The grains were then milled using a Foss Cyclotec 1093™ (FOSS, Barcelona, Spain) mill equipped with a 0.5 mm screen. Finally, the flour was immediately stored at -20 °C in the dark until analysis.

III.4.3. Determination of β-glucans and Arabinoxylans

Total β-glucan and arabinoxylan contents were measured, respectively, by the mixed-linkage β-glucan assay (K-BGLU) (β-Glucan Assay Kit) and D-xylose assay (K-XYLOSE) (D-Xylose Assay Kit) kits from Megazyme (Wicklow, Ireland), according to the manufacturer's instructions.

III.4.4. Determination of Anthocyanins

Analysis of anthocyanins was carried out according to Abdel-Aal and Hucl (1999). The absorbance was read at 535 nm using a Multiscan GO spectrophotometer (Thermo Scientific, Vantaa, Finland). The anthocyanins were quantified with a standard calibration curve obtained for the Cyanidin-3-glucoside and expressed as µg Cy-3-glu/g.

III.4.5. Determination of Free and Bound Phenolic Compounds

Free and bound phenolic compounds were determined the second year, using the extraction method reported by Martínez et al. (2018). Both fractions were then analyzed using the spectrophotometric Folin−Ciocalteu method adapted to a microplate format by Bobo-García et al. (2014). The absorbance was read at 760 nm using a Multiscan GO spectrophotometer (Thermo Scientific, Vantaa, Finland). The phenolic compounds were quantified with a standard calibration curve obtained for the Gallic acid equivalent (GAE) and expressed as mg GAE/g.

III.4.6. Determination of Antioxidant Capacity

Antioxidant capacity (AC) was also measured in the second year with the oxygen radical absorbance capacity (ORAC) assay, according to Huang et al. (2002). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as the control. Antioxidant capacity was expressed as µmols Trolox equivalent/g.

III.4.7. Statistical Analysis.

All bioactive compound contents were referred to grain weight, either as a mg/g, µg/g and µmols/g depending on their final concentration. In order to characterize the deposition profile of the different bioactive compounds, the Specialized Modelling procedure in JMP14 Pro (SAS institute Inc., Cary NC) was used to fit the average of two blocks for each genotype x year x environment combinations simultaneously, using growing degree-days after anthesis (GDA), determined using maximum and minimum daily temperature with a based temperature of 0 °C, as thermal time. Nonlinear standard 3-parameter logistic curve and alternative polynomial linear models were compared. The best model was identified using the minimum value of the Akaike information criterion. JMP14 directly provides the estimates and standard errors for the 3-parameters of the logistic sigmoid for each curve: growth rate, inflection point and maximum value. As the 3-parameter logistic curve is symmetrical around its inflection point, we approximated duration and its standard error by multiplying the inflection point and its standard error by 1.9. In other words, we defined duration as the thermal time needed to increase the content from 5% to 95% of the final value. In order to study the dynamics of the contents of the different bioactive compounds during the course of grain filling, whenever the logistic model was selected, we compared the estimates for the three parameters determined for each of the curves directly by means of simple analysis of variance. As the use of standard errors as weights did not alter the results, simpler unweighted ANOVA models were preferred. The number of curves did not allow for a full three-factor factorial expansion. Therefore, two- and three-way interactions, with the exception of the fixed genotype x environments (i.e., stressed *vs*. control conditions) interaction, were pooled into a single error term. Deposition patterns for bioactive compounds following a linear polynomial model were directly studied by incorporating first- or higher-order thermal time terms into a standard covariance linear model.

III.5. Conclusions

Barley cultivated in a heat-stressed area, specifically under Mediterranean conditions, is a valuable source of dietary fiber, phenolic compounds and antioxidant capacity for cereal-based healthy food products. In general, the contents of the bioactive compounds were determined more by the genotype than by the environment. Induced late high-temperature stress reduced final grain weight, did not affect the β-glucans or arabinoxylan contents and increased the phenolic compounds, as well as their antioxidant capacity, especially for Annapurna and Hispanic. However, future research would be necessary to determine whether the structure of some of these bioactive compounds is affected during grain accumulation in high-temperature stress-prone areas, as it can influence the quality of the barley-based products. The concentration of bioactive compounds changed differentially throughout grain filling, depending on the development time when they were synthesized. The deposition patterns for dietary fiber followed that of grain weight. Annapurna needed less thermal time to increase the grain weight and β-glucans content from 5% to 95% of the final value during grain filling. Anthocyanins reached a maximum before the end of grain filling. The rate of anthocyanins deposition changed under control and stress conditions, likely due to the plastic covering used to increase the temperature that reduced the solar radiation, decreasing the anthocyanin content in the colored genotype. Free and bound phenols constantly decreased as the grains developed, suggesting that they are synthesized in early stages. However, it was observed that the rate of deposition of bound phenols was more sensitive to genotypic changes; Hindukusch, the purple genotype, behaved differently than the other genotypes. Overall, the antioxidant

capacity decreased over time, but the decay observed in the control plots was steeper than for the stress-induced ones. These results support food barley cultivation in high-temperature stress-prone areas, as some bioactive compound and antioxidant capacity will increase, regardless of the smaller size grains. Furthermore, if a market develops for food barley ingredients, early harvesting of non-mature grains should be considered to maximize antioxidant capacity.

III.6. Author Contributions

Conceptualization, I.R., R.S., and M.M.S.; methodology, R.S., I.R., and M.P.R.; field and laboratory analyses, M.M.S. and E.P.; statistical analyses, M.M.S. and I.R.; data curation, I.R.; writing—original draft, M.M.S.; writing—review and editing I.R., R.S., M.M., and M.P.R.; supervision I.R. and R.S.; funding acquisition I.R. and M.M. All authors have read and agreed to the published version of the manuscript.

III.7. Abbreviations

AC: Antioxidant Capacity; Cy-3-glu, Cyanidin-3-glucoside; DC, Decimal Code; GAE, Gallic Acid Equivalent, GDA, Growing Degree-days accumulated after Anthesis; GW, Grain Weight; ORAC, Oxygen Radical Absorbance Capacity; PAL, Phenylalanine Ammonia Lyase.

III.8. Funding

This work was funded by project AGL 2015-69435-C3-1 from the Spanish Ministry of Economy and Competitiveness. Mariona Martínez-Subirà was supported by a pre-doctoral fellowship (BES-2016-078654/AGL 2015-69435-C3-1).

III.9. Conflicts of Interest

The authors declare no conflicts of interest.

III.10. Supplementary information

Table III.S.1. Grain characteristics and average final contents of grain weight (GW), β-glucans, arabinoxylans, total phenolic compounds and antioxidant capacity (AC) of four barley genotypes during two consecutive seasons of the control samples without heat stress.

Genotype		Endosperm	Hull	Row	Colour	β-glucan content	Average final contents of two growing seasons				
							GW (mg)	β-glucan (mg/ g)	Arabinoxylan (mg/ g)	Phenolic compounds (mg/ GAE g)	AC (µmols Trolox/ g)
Annapurna	Spanish variety	Waxy	Naked	2	Yellow	High	51 a	79 a	55 a	4.6 bc	111 b
Hindukusch	Afghan landrace	Non-waxy	Partially naked	2	Purple	Medium	47 ab	68 b	55 a	5.7 a	140 a
Hispanic	European variety	Non-Waxy	Hulled	2	Yellow	Low	52 a	50 c	41 c	4.2 c	91 c
Tamalpais	North American variety	Non-Waxy	Naked	6	Yellow	High	44 b	80 a	45 b	4.9 b	110 a

Results are presented as mean. Mean with a column followed by different letters indicate significant differences; Tukey-Kramer (α = 0.05).

III.11. References

Abdel-Aal ESM, Hucl P (1999) A rapid method for quantifying total anthocyanins in blue aleurone and purple pericarp wheats. Cereal Chem. 76.350–354.

Ahmed N, Maekawa M, Noda K (2009) Anthocyanin accumulation and expression pattern of anthocyanin biosynthesis genes in developing wheat coleoptiles. Biol. Plant. 53:223–228.

Biliaderis CG, Lazaridou A (2009) Cereal non-starch polysaccharides as functional food ingredients: Structure-physical proper-ties relations. PTEP 13:197–201.

Bobo-García G, Davidov-Pardo G, Arroqui C, Vírseda P, Marín-Arroyo MR, Navarro M (2014) Intra-laboratory validation of microplate methods for total phenolic content and antioxidant activity on polyphenolic extracts, and comparison with conventional spectrophotometric methods. J. Sci. Food Agric. 95:204–209.

Bustos DV, Riegel R, Calderini DF (2012) Anthocyanin content of grains in purple wheat is affected by grain position, assimilate availability and agronomic management. J. Cereal Sci. 55:257–264.

De Arcangelis E, Messia MC, Marconi E (2019) Variation of polysaccharides profiles in developing kernels of different barley cultivars. J. Cereal Sci. 85:273–278.

De Graaff P, Govers C Wichers HJ, Debets R (2018) Consumption of β-glucans to spice up T cell treatment of tumors: A re-view. Expert Opin. Biol. Ther. 18:1023–1040.

Dell'Aversana E, Hessini K, Ferchichi S, Fusco GM, Woodrow P, Ciarmiello LF, Abdelly C, Carillo, P (2021) Salinity Duration Differently Modulates Physiological Parameters and Metabolites Profile in Roots of Two Contrasting Barley Genotypes. Plants 10:307.

Dias AS, Lidon FC (2009) Evaluation of Grain Filling Rate and Duration in Bread and Durum Wheat, under Heat Stress after Anthesis. J. Agron. Crop. Sci. 195:137–147.

Dodig D, Kandi V, Zori M, Nikoli-Dori E, Nikoli A, Mutavdži B, Perov D, Šurlan-Momirovi G (2018) Comparative kernel growth and yield components of two- and six-row barley (*Hordeum vulgare*) under terminal drought simulated by defoliation. Crop. Pasture Sci. 69:1215–1224.

D-Xylose Assay Kit (K-XYLOSE). Megazyme (Wicklow, Ireland). Available online: https://www.megazyme.com/documents/Booklet/K-XYLOSE_DATA.pdf [10 March 2021].

EFSA, 2011. Scientific opinion on the substantiation of health claims related to β-glucans from oats and barley and maintenance of normal blood LDL-cholesterol concentrations (ID 1236, 1299), increase in satiety leading to a reduction in energy intake (ID 851, 852), reduction of post-prandial glycaemic responses (ID 821, 824), and "digestive function" (ID 850) pursuant to Article 13(1) of Regulation (EC) No 1924/2006. EFSA J. 2011, 9, 2207. Available online: http://www.efsa.europa.eu/en/efsajournal/pub/2207 [8 June 2020].

Egli DB (2006) The role of seed in the determination of yield in grain crops. Aust. J. Agric. Res. 57:1237–1247.

Elía M, Slafer GA, Savin R (2018) Yield and grain weight responses to post-anthesis increases in maximum temperature under field grown wheat as modified by nitrogen supply. Field Crops Res. 221:228–237.

Ezquer I, Salameh I, Colombo L, Kalaitzis P (2020) Plant Cell Walls Tackling Climate Change: Biotechnological Strategies to Improve Crop Adaptations and Photosynthesis in Response to Global Warming. Plants 9:212.

FDA, 2008. Food Labeling: Health Claims; Soluble Dietary Fiber from Certain Foods and Coronary Heart Disease. [Docket No. FDA-2008-P0090]. Available: https://pubmed.ncbi.nlm.nih.gov/12361061/ [8 June 2020].

Gonzalez-San José ML, Diez C (1992) Relationship between anthocyanins and sugars during the ripening of grape berries. Food Chem. 43:193–197.

Guo R, Yuan G, Wang Q (2011) Effect of sucrose and mannitol on the accumulation of health promoting compounds and the activity of metabolic enzymes in broccoli sprouts. Sci. Hortic. 128, 159–165.

Huang D, Ou B, Hampsch-Woodill M, Flanagan JA, Prior RL (2002) High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. J. Agric. Food Chem. 50:4437–4444.

Idehen E, Tang Y, Sang S (2017) Bioactive phytochemicals in barley. J. Food Drug Anal. 25:148–161.

IPCC, 2013. Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change; TF Stocker, D Qin, G-K Plattner, MMB Tignor, SK Allen, J Boschung, A Nauels, Y Xia, V Bex, PM Midgley, (Ed).; Cambridge University Press: Cambridge, UK; New York, NY, USA, 2013; p. 1535.

Knievel DC, Abdel-Aal ESM, Rabalski I, Nakamura T, Hucl P (2009) Grain color development and the inheritance of high anthocyanin blue aleurone and purple pericarp in spring wheat (*Triticum aestivum* L.). J. Cereal Sci. 50:113–120.

Ko J, Ng CT, Jeong S, Kim J-H, Lee B, Kim H-Y (2019) Impacts of regional climate change on barley yield and its geo-graphical variation in South Korea. Int. Agrophys. 33:81–96.

Li J, Lin X, Chen A, Peterson T, Ma K, Bertzky M, Ciais P, Kapos V, Peng C, Poulter B (2013) Global Priority Conservation Areas in the Face of 21st Century Climate Change. PLoS ONE 8:e54839.

Loss SP, Kirby EJM, Siddique KHM, Perry MW (1989) Grain growth and development of old and modern Australian wheats. Field Crop. Res. 21:131–146.

Ma D, Li Y, Zhang J, Wang C, Qin H, Ding H, Xie Y, Guo T (2016) Accumulation of phenolic compounds and expression profiles of phenol acid biosynthesis-related genes in developing grains of white, purple and red wheat. Front. Plant. Sci. 7:528.

MacLeod LC, Duffus CM (1988) Reduced starch content, sucrose synthase activity in developing endosperm of barley plants grown at elevated temperatures. Aust. J. Plant. Physiol. 15:367–375.

Martínez M, Motilva MJ, López de las Hazas MC, Romero MP, Vaculova K, Ludwig IA (2018) Phytochemical composition and β-glucan content of barley genotypes from two different geographic origins for human health food production. Food Chem. 245, 61–70.

Martínez-Subirà M, Romero MP, Moralejo M, Macià A, Puig E, Savin R, Romagosa I (2021) Post-anthesis thermal stress induces differential accumulation of bioactive compounds in field-grown barley. J. Sci. Food Agric.

McCallum JA, Walker JR (1991) Phenolic biosynthesis during grain development in wheat (*Triticum aestivum* L.) III. Changes in hydroxycinnamic acids during grain development. J. Cereal Sci. 13, 161–172.

Molina-Cano JL, Francesch M, Perez Vendrell AM, Ramo T, Voltas J, Brufau J (1997) Genetic and environmental variation in malting and feed quality of barley. J. Cereal Sci. 25:37–47.

Özkaya B, Turksoy S, Özkaya H, Baumgartner B, Özkeser İ, Köksel H (2018) Changes in the functional constituents and phytic acid contents of firiks produced from wheats at different maturation stages. Food Chem. 246:150–155.

Pandey KB, Rizvi SI (2009) Plant polyphenols as dietary antioxidants in human health and disease. Oxid. Med. Cell Longev. 2:270–278.

Rakszegi M, Lovegrove A, Balla K, Láng L, Bedo Z, Veisz O, Shewry PR (2014) Effect of heat and drought stress on the structure and composition of arabinoxylan and β-glucan in wheat grain. Carbohydr. Polym. 102:557–565.

Savin R, Nicolas ME (1996) Effects of short periods of drought and high temperature on grain growth and starch accumulation of two malting barley cultivars. Aust. J. Plant. Physiol. 23:201–210.

Schulman AH, Runeberg-Roos P, Jääskeläinen M (2000) Grain filling and starch synthesis in barley. In: AK Gupta, N Kaur (Ed.), Carbohydrate Reserves in Plants-Synthesis and Regulation, 1st ed.; Elsevier Science B.V.: Amsterdam, New York The Netherlands, 2000; pp. 147-167.

Servei Meteorologic de Catalunya. Available online: https://www.meteo.cat [3 September 2020].

Shamloo M, Babawale EA, Furtado A, Henry RJ, Eck PK, Peter JH, Jones PJH (2017) Effects of genotype and temperature on accumulation of plant secondary metabolites in Canadian and Australian wheat grown under controlled environments. Sci. Rep. 7, 9133.

Shao Y, Xu F, Sun X, Bao J, Beta T (2014) Phenolic acids, anthocyanins, and antioxidant capacity in rice (*Oryza sativa* L.) grains at four stages of development after flowering. Food Chem. 143:90–9691.

Sharma A, Babar Shahzad B, Abdul Rehman A, Renu Bhardwaj R, Marco Landi M, Zheng B (2019) Response of Phenylpropanoid Pathway and the Role of Polyphenols in Plants under Abiotic Stress. Molecules 24.2452.

Silvestro RD, Loreto AD, Bosi S, Bregola V, Marotti I, Benedettelli S, Segura-Carretero A, Dinelli G (2016) Environment and genotype effects on antioxidant properties of organically grown wheat varieties: A 3-year study. J. Sci. Food Agric. 97:641–664.

Solfanelli C, Poggi A, Loreti E, Alpi A, Perata P (2006) Sucrose-specific induction of the anthocyanin biosynthetic pathway in Arabidopsis. Plant. Physiol. 140:637–646.

Suriano S, Iannucci A, Codianni P, Fares C, Russo M, Pecchioni N, Marciello U, Savino M (2018) Phenolic acids profile, nutritional and phytochemical compounds, antioxidant properties in colored barley grown in southern Italy. Int. Food Res. J. 113:221–233.

Swanston JS, Ellis RP, Perez -Vendrell A, Voltas J, Molina-Cano JL (1997) Patterns of barley grain development in Spain and Scotland and their implications for malting quality. Cereal Chem. 74:456–461.

Tsormpatsidis E, Henbest RGC, Davis FJ, Battey NH, Hadley P, Wagstaffe A (2008) UV irradiance as a major influence on growth, development and secondary products of commercial importance in Lollo Rosso lettuce 'Revolution' grown under polyethylene films. Environ. Exp. Bot. 63:232–239.

Verspreet J, Hemdane S, Dornez E, Cuyvers S, Pollet A, Delcour JA, Courtin CM (2013) Analysis of Storage and Structural Carbohydrates in Developing Wheat (*Triticum aestivum* L.) Grains Using Quantitative Analysis and Microscopy. J. Agric. Food Chem. 61:9251–9259.

Wilson SM, Burton RA, Collins HM, Doblin MS, Pettolino FA, Shirley N, Fincher GB, Bacic A (2012) Pattern of Deposi-tion of Cell Wall Polysaccharides and Transcript Abundance of Related Cell Wall Synthesis Genes during Differentiation in Barley Endosperm. Plant. Physiol 159:655–670.

Wu G, Johnson SK, Bornman JF, Bennett SJ, Clarke MW, Singh V, Fang Z (2016) Growth temperature and genotype both play important roles in sorghum grain phenolic composition. Sci. Rep. 6:21835.

Zadoks JC, Chang TT, Konzak CF (1974) A decimal code for the growth stages of cereals. Weed Res. 14:415–421.

Zhang MW, Zhang RF, Zhang FX, Liu RH (2010) Phenolic profiles and antioxidant activity of black rice bran of different commercially available varieties. J. Agric. Food Chem. 58:7580–7587.

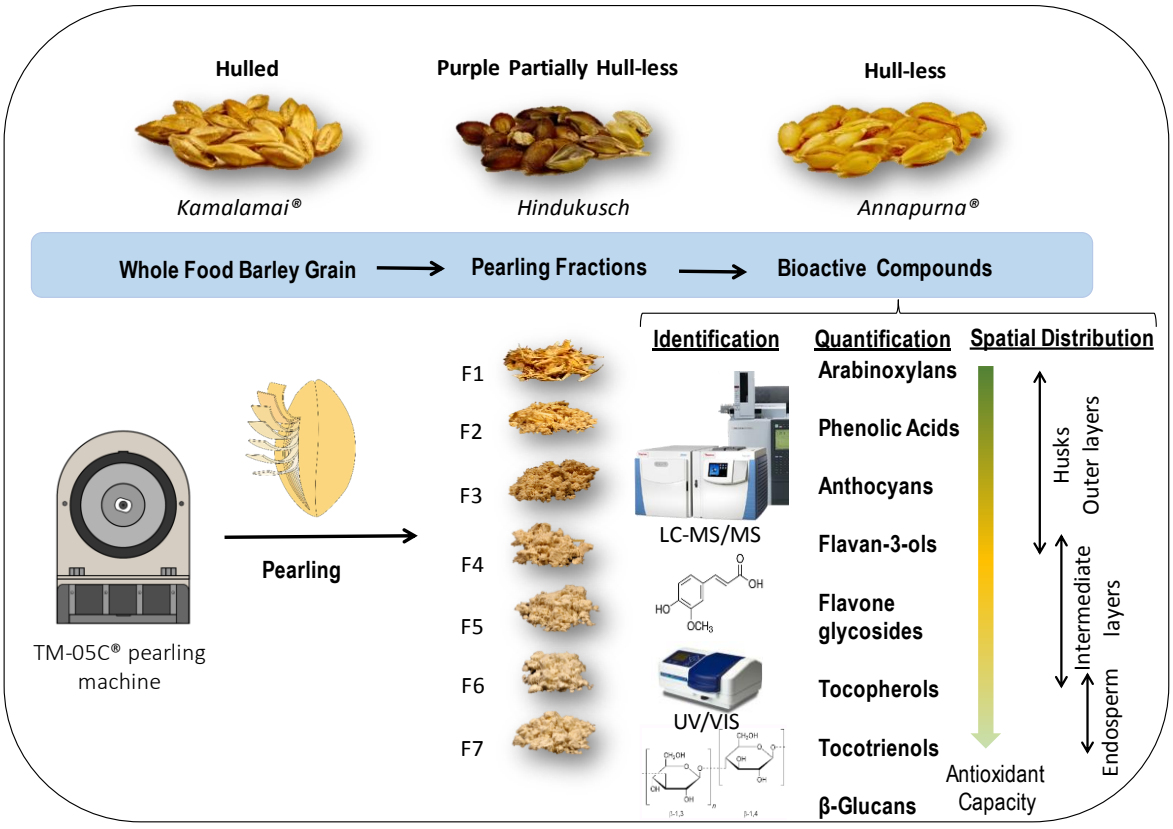
Zhu Y, Li T, Fu X, Abbasi AM, Zheng B, Liu RH (2015) Phenolics content, antioxidant and antiproliferative activities of dehulled highland barley (*Hordeum vulgare* L.). J. Funct. Foods. 19:439–450.

β-Glucan Assay Kit (Mixed Linkage) (K-BGLU). Megazyme (Wicklow, Ireland). Available online: https://www.megazyme.com/documents/Booklet/K-BGLU_DATA.pdf [10 March 2021]

Chapter IV

Bioactive Compounds and Antioxidant Capacity in Pearling Fractions of Hulled, Partially Hull-Less and Hull-Less Food Barley Genotypes*

M. Martínez-Subirà, M.-P. Romero, A. Macià, E. Puig, I. Romagosa and M. Moralejo



Graphical abstract

* Martínez-Subirà, M.; Romero, M.P.; Macià, A.; Puig, E.; Romagosa, I.; Moralejo, M. Bioactive Compounds and Antioxidant Capacity in Pearling Fractions of Hulled, Partially Hull-Less and Hull-Less Food Barley Genotypes. Foods. 2021, 10(3), 565; https://doi.org/10.3390/foods10030565

foods

MDPI

Article

Bioactive Compounds and Antioxidant Capacity in Pearling Fractions of Hulled, Partially Hull-Less and Hull-Less Food Barley Genotypes

Mariona Martínez-Subirà, María-Paz Romero, Alba Macià, Eva Puig, Ignacio Romagosa and Marian Moralejo *

AGROTECNIO-CERCA Center, University of Lleida, Av. Rovira Roure 191, 25198 Lleida, Spain; mariona.martinez@udl.cat (M.M.-S.); mariapaz.romero@udl.cat (M.-P.R.); alba.macia@udl.cat (A.M.); eva.puig@udl.cat (E.P.); ignacio.romagosa@udl.cat (I.R.) * Correspondence: marian.moralejo@udl.cat; Tel.: +34-97370-2858

Citation: Martínez-Subirà, M.; Romero, M.-P.; Macià, A.; Puig, E.; Romagosa, I.; Moralejo, M. Bioactive Compounds and Antioxidant Capacity in Pearling Fractions of Hulled, Partially Hull-Less and Hull-Less Food Barley Genotypes. Foods 2021, 10, 565. https://doi.org/10.3390/foods10030565

Academic Editors: Marina Russo and Francesco Cacciola

Received: 3 February 2021
Accepted: 4 March 2021
Published: 9 March 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Abstract: Three food barley genotypes differing in the presence or absence of husks were sequentially pearled and their fractions analyzed for ash, proteins, bioactive compounds and antioxidant capacity in order to identify potential functional food ingredients. Husks were high in ash, arabinoxylans, procyanidin B3, prodelphinidin B4 and p-coumaric, ferulic and diferulic bound acids, resulting in a high antioxidant capacity. The outermost layers provided a similar content of those bioactive compounds and antioxidant capacity that were high in husks, and also an elevated content of tocols, representing the most valuable source of bioactive compounds. Intermediate layers provided high protein content, β-glucans, tocopherols and such phenolic compounds as catechins and bound hydroxybenzoic acid. The endosperm had very high β-glucan content and relative high levels of catechins and hydroxybenzoic acid. Based on the spatial distribution of the bioactive compounds, the outermost 30% pearling fractions seem the best option to exploit the antioxidant capacity of barley to the full, whereas pearled grains supply β-glucans enriched flours. Current regulations require elimination of inedible husks from human foods. However, due to their high content in bioactive compounds and antioxidant capacity, they should be considered as a valuable material, at least for animal feeds.

Keywords: Whole barley flour; pearling fractions; proteins; β-glucans; arabinoxylans; tocols; phenolic compounds; antioxidant capacity; functional food

IV.1. Introduction

Barley (*Hordeum vulgare* L.) is the fourth most cultivated cereal in the world after maize, wheat and rice (Faostat). It is widely used for feed and malt, with limited consumption as human food in some specific regions, such as the Maghreb and the high plateaus of the Himalayas. However, in recent years, it has attracted growing interest worldwide due to the health promoting properties of its bioactive compounds. In terms of health, several reports have demonstrated the positive effect of barley on the glycemic index, cholesterol and heart diseases (Baik and Ullrich, 2008). This is mainly due to the presence of β-glucans (2–11% d.w.), a dietary fiber component for which health claims have been approved by the US Food and Drug Administration and the European Food Safety Authority (FDA, 2008; EFSA, 2011). Additional beneficial effects have been described for β-glucans. These include their properties as enhancers of the immune system against infectious diseases and some types of cancer, and also as a key modulator of the composition and activity of human microbiota (Hong et al., 2004; De Graaff et al., 2018; Bai et al.,2019). Arabinoxylans are the second major component of barley dietary fiber (2–9% d.w.). Their proven health benefits include effects on the postprandial glucose response, cholesterol metabolism and immune response (Izydorczyk and Dexter, 2008; Fadel et al., 2018). In barley, arabinoxylans are particularly associated with antioxidant activity due to the presence of phenolic acids linked to their structure (Malunga and Beta, 2015). Barley is also a significant source of antioxidant compounds (Gangopadhyay et al., 2016; Martínez et al., 2018). It contains more vitamin E than most cereals (17–49 μg/g d.w.), this being in the form of α-tocopherol and α-tocotrienol. Furthermore, barley contains high levels of phenolic compounds such as phenolic acids and flavan-3-ols which can be found free or ester-bound to the fiber. Flavan-3-ols like catechins, procyanidins and prodelphinidins are the most abundant free phenols, while phenolic acids such as ferulic, p-coumaric and vanillic are the major constituents within the bounds. Anthocyanins are also present in considerable concentrations in some barley genotypes with colored kernels. All these phenolic compounds are considered potent antioxidants, free radical scavengers and inhibitors of lipid peroxidation (Abdel-Aal et al., 2012; Suriano et al., 2018). In addition, preclinical studies and clinical trials have shown that polyphenols could greatly modulate the gut microbiota, thus favoring the growth of potential beneficial organisms and simultaneously inhibiting pathogenic bacteria (Corrêa et al., 2019).

Barley grains may differ in important morphological characters. These include being hulled (covered), when the husks adhere to the caryopsis, partially hull-less (skinned), when a partial loss of the husks occurs, and hull-less (naked), when the husks are freely threshed at harvest, the latter being what is preferred for human consumption; grains from two or six rowed spikes or grains with colors such black, blue or purple, alternative to yellow. Based on the grain composition, barley is further classified as normal, high amylopectin (waxy) or high amylose starch types, high β-glucan, high lysine, and proanthocyanidin free. All these types of grain differ widely in their physical and compositional characteristics and, accordingly, are processed differently and used for different commercial purposes.

The bioactive compounds are not uniformly distributed across the barley grains. It is known that arabinoxylans, tocotrienols and phenolics are mainly located in the outer layers, tocopherols in the germ, and β-glucans in the endosperm (Panfili et al., 2008). Thus, physical processes like pearling, an abrasive technique that gradually removes grain layers to obtain polished grain and by-products, allow favorable separation of fractions enriched in specific compounds and these can be used as functional ingredients. Several fold enrichment of antioxidant compounds has been described in

barley pearled fractions (Badea et al., 2018; Irakli et al., 2020) that have been used to improve the nutritional value of such wheat-based products as cookies, pasta and bread (Marconi et al., 2000; Sharma and Gujral, 2014; Martínez-Subirà et al., 2020).

In recent years, increased efforts have been carried in a few countries to release new specific barley varieties for human consumption and for the food industry. In our food barley breeding program, we aim to produce varieties rich in β-glucans as well as antioxidant compounds adapted to the Spanish agroclimatic conditions. Three distinct high β-glucan barley genotypes from our program, differing in the type of grain (Kamalamai, hulled; Hindukusch, partially hull-less; Annapurna, hull-less) were selected to identify specific potential ingredients for the functional food industry. Thus, the main objectives of this work were as follows: (1) to analyze β-glucans, arabinoxylans, tocols, phenolic compounds and antioxidant capacity in the whole flours and pearling fractions of different types of barley grains; (2) to identify pearling fractions to be used potentially as functional ingredients. This research could provide further knowledge about the spatial distribution of a large number of bioactive compounds in barley pearling fractions, since most published works have focused on changes in various bioactive compounds separately (Panfili et al., 2008; Madhujith and Shahidi, 2009; Badea et al., 2018). To the best of our knowledge, this is the first time that such an integrative study of the major health-promoting components in barley genotypes specifically bred for human food has been explored.

IV.2. Materials and Methods

IV.2.1. Plant Material

- Kamalamai®: registered Spanish variety, hulled, two rowed, normal endosperm (semillas Batlle SA).
- Hindukusch: Afghan landrace, naked but often suffering from grain skinning, two rowed, normal endosperm and purple grain used as parent in our crosses.
- Annapurna®: registered Spanish variety, hull-less, two rowed, waxy endosperm (semillas Batlle SA).

The three genotypes were cultivated under similar conditions in Bell-lloc d'Urgell, Lleida (Spain) during the 2018-2019 growing season.

IV.2.2. Whole Flours and Pearling Fractions

Grain with size above 2.5 mm was screened for this study using a stainless-steel mesh. Six pearling fractions from each genotype were obtained by sequential processing of grain using theTM-05C pearling machine (Satake Corporation, Hiroshima, Japan) at 1060 rpm. The grains were initially pearled to remove the 5% of the original grain weight that resulted in the first fraction F1 (0-5% w/w). The remained grains were pearled to remove the second fraction F2 (5-10%), and then the process was repeated to get fractions F3 (10-15%), F4 (15-20%), F5 (20-25%), F6 (25-30%), and the residual 70% pearled grain F7 (30-100% w/w). After each pearling session, the pearling machine was cleaned to avoid mixtures between fractions. Fractions, pearled grains and whole grains were ground in a Foss Cyclotec 1093™ mill equipped with a 0.5-mm screen (Foss Iberia, Barcelona, Spain) prior to chemical analyses.

IV.2.3. Protein and Ash Content

The protein content was done according to the Kjeldahl method in a Kjeltec system I (Foss Tecator AB, Höganäs, Sweden) using the conversion factor of 5.7. The ash content was determined in a muffle furnace according to the AOAC Official Method 942.05 (Thiex et al., 2012).

IV.2.4. β-Glucan and Arabinoxylan Content

The β-glucan and arabinoxylan contents were determined by means of the mixed-linkage β-glucan assay (K-BGLU) and D-xylose assay (K-XYLOSE) kits from Megazyme (Wicklow, Ireland).

IV.2.5. Tocols Content

Tocopherols and tocotrienols (α-, β-, γ-, and δ-isomers) were quantified by high performance liquid chromatography (HPLC) coupled to a fluorescence detector. One gram of each barley genotype was extracted three times with 10 mL n-hexane and the extract collected after centrifuging at 9000× g for 10 min. The supernatants were pooled, reduced to dryness under a flow of nitrogen, and reconstituted in 1 mL of n-hexane. Normal phase HPLC with fluorescence detection (excitation 292 nm, emission 325 nm) was used to analyze tocopherols and tocotrienols. Aliquots of 50 µL were injected into the HPLC system following the chromatographic conditions described by Martínez-Subirà et al. (2018). Tocopherols and tocotrienols isomers were quantified with external standard curves. Results were expressed as µg/g dry sample.

IV.2.6. Phenolic Compounds (PCs) and Anthocyanin Analysis by UPLC-MS/MS

Free phenolic compounds were extracted three times by adding 1 mL of 79.5% methanol, 19.5% Milli Q water, and 1% formic acid solution to 150 mg of barley flours. The samples were sonicated for 30 s and centrifuged at 9000× g for 10 min. The supernatants from each extraction were pooled and filtered through 0.22 µm polyvinylidene fluoride (PVDF) filter discs before chromatographic analysis. The residue was subjected to alkaline hydrolysis by adding 6 mL of 2 mol/L NaOH to obtain bound phenols. The samples were left over-night at room temperature for complete hydrolysis. Then, they were sonicated for 1 min and centrifuged at 9000× g for 10 min; the supernatant was acidified with HCl 37% (w/w) to pH 2. A total of 350 µL of supernatant was mixed with phosphoric acid 10 min, centrifuged at 9000× g, and subjected to µSPE clean-up according to Serra et al. (2013). Briefly, the micro-cartridges were pre-conditioned with acidified water (pH 2) and methanol. The samples were loaded onto the µSPE and subsequently washed with water and water/methanol 95/5 (v/v). The PCs were eluted with methanol, and 2.5 µL of the eluate was directly analyzed by liquid chromatography. The extracts were analyzed by Ac Quity Ultra-Performance TM liquid chromatography coupled to a tandem mass spectrometer (UPLC-MS/MS), equipped with the analytical column Ac Quity BEH C18 (100 mm × 2.1 mm i. d., 1.7 µm) and the Van Guard TM Pre-Column Ac Quity BEH C 18 (5 mm × 2.1 mm, 1.7 µm), all from Waters, Milford, MA, USA. The mobile phase was 0.2% (v/v) acetic acid and acetonitrile for the phenolic compounds (PCs), and 10% acetic acid (v/v) and acetonitrile for the anthocyanins. The UPLC system was coupled to a triple quadrupole detector mass spectrometer from Waters equipped with a Z-spray electrospray interface for ionization, operating in the negative mode [M−H]− for the PCs and the positive mode [M−H]+ for the anthocyanins. Quantification was based on a 0.02–25 ng calibration curve of commercially available standards, and the results were expressed as µg/g dry sample. A linear response was obtained for all standards and tested by linear regression analysis. The limits of detection (LOD) ranged from 0.007 to 0.09 ng and the limits of quantification (LOQ) from 0.02 to 0.30 ng.

IV.2.7. Antioxidant Capacity (AC) Analysis

The Oxygen Radical Absorbance Capacity (ORAC) of both the free and bound extracts was measured as described Huang et al. (2002). The antioxidant capacity (AC) was determined using the FLUO star OPTIMA fluorescence reader (BMG Labtech, Offenburg, Germany) in a 96-well polystyrene microplate controlled by the OPTIMA 2.10 R2 software. Changes in fluorescence were measured under controlled temperature (37 °C) in a reader with fluorescence filters with 485 nm excitation and 520 nm emission wavelengths. Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) was used as control, with one ORAC unit being equal to the antioxidant protection given by 1 µmol Trolox. The results were expressed as µmols of Trolox-equivalents per 100 g of dry sample.

IV.2.8. Statistical Analysis

All statistical analyses were conducted using JMP®Pro14 (SAS institute Inc., Cary, NC, USA). Analytical measurements were carried out in triplicate and the results were presented as mean values. Data was checked for normality and for homoscedasticity of variances based on a number of diagnostic tools, such as residual plots, Box-Cox's Lambda and Levene's test of equality of variances, provided by JMP. When needed, a logarithmic transformation was used and indicated in the text. For multiple comparisons, Tukey–Kramer's honestly-significant-difference tests (HSD) (α = 0.05) were conducted once the corresponding ANOVA F-tests were found significant. Principal Component Analysis (PCA) was used to graphically represent the association between fractions, genotypes and bioactive compounds using standardized data.

IV.3. Results and Discussion

IV.3.1. Ash and Protein Contents

The ash and protein contents of the whole flours and pearling fractions are shown in Table IV.1. The hulled genotype Kamalamai showed the highest ash content and Hindukush, the partially hull-less genotype, the highest protein amount. The distribution through the grain showed a progressive decrease in the ash content from the first pearling fraction F1 of the three genotypes toward the endosperm. This was because the mineral components are mainly localized in the outer layers of the kernel. The protein content was the lowest in the initial surface F1 fraction of the Kamalamai, which mainly correspond to husks, whereas the highest content was detected in the middle fraction F4 of both genotypes containing husks, and F3 of the hull-less Annapurna genotype. These results were in accordance to that observed by other authors on barley fractions obtained by pearling or roller-milling processes (Panfili et al., 2008; Blandino et al., 2015).

Table IV.1. Ash and protein contents in whole barley flours and pearling fractions.

	Ash (%)						Protein (%)					
	Kamalamai		**Hindukush**		**Annapurna**		**Kamalamai**		**Hindukush**		**Annapurna**	
Whole flour	1.97	a	1.68	a	1.48	b	14.62	b	15.26	a	14.83	ab
Fractions												
F1	6.64	a	6.52	a	5.16	a	7.77	g	10.54	e	19.97	e
F2	5.60	b	5.75	b	4.74	b	17.88	e	17.17	d	25.63	c
F3	5.00	c	5.86	b	3.81	c	24.18	d	23.10	c	27.88	a
F4	3.93	d	5.09	c	3.23	d	26.72	a	27.57	a	27.34	b
F5	3.17	e	3.94	d	2.26	e	26.11	b	27.34	a	25.33	c
F6	2.50	f	2.76	e	1.77	f	25.21	c	26.81	b	23.11	d
F7	0.87	g	0.76	f	1.13	g	12.80	f	10.54	f	11.93	f
SED	*0.04*		*0.05*		*0.03*		*0.13*		*0.11*		*0.14*	

Results are presented as the mean. Means within a column followed by different letters indicate significant differences; (Tukey–Kramer`s HSD for α = 0.05) SED: standard error of the difference between means.

IV.3.2. β-Glucans, Arabinoxylans and Tocols Contents

In barley, the β-glucan content depends on genetic and environmental factors as well as on the interaction between these (Swanston et al., 1997; Izydorczyk and Dexter, 2008). In our work, the three barley genotypes were high in β-glucans with amounts ranging from 8.3 to 9.5 g/100 g (Table IV.2). Annapurna had the highest β-glucan content while both genotypes containing husks showed similar values. These results agree with earlier studies which described higher β-glucan levels in hull-less and waxy genotypes like Annapurna rather than in hulled barley with normal endosperm (Andersson et al., 2008; Baik and Ullrich,2008). The distribution of this fiber component through the grain is shown in Figure IV.1. β-glucans increased gradually from the outer to inner layers of the grains in accordance with previous findings (Izydorczyk et al., 2014; Blandino et al., 2015). The lowest concentrations were detected in the outer fraction F1 of the three genotypes and progressively increased until F4 of Annapurna, F5 of Kamalamai, and F6 of Hindukusch, after which they remained constant. The net effect was that removing the 30% outer fractions of Kamalamai and Hindukusch increased the β-glucan content by 9% and 6%, respectively, while this increase was only 3% in the hull-less genotype Annapurna.

Table IV.2. β-glucans, arabinoxylans and tocols contents in whole barley flours.

	Kamalamai				Hindukusch				Annapurna			
β-glucans (g/100g)	8.41	±	0.09	b	8.26	±	0.07	b	9.46	±	0.10	a
Arabinoxylans (g/100g)	5.52	±	0.27	b	6.60	±	0.44	a	5.52	±	0.19	b
α-Tocopherol (µg/g)	8.32	±	0.21	a	7.53	±	0.22	a	6.22	±	0.19	b
β-Tocopherol (µg/g)	0.03	±	0.01	a	0.03	±	0.01	a	0.03	±	0.01	a
γ-Tocopherol (µg/g)	1.58	±	0.00	a	1.08	±	0.01	c	1.21	±	0.01	b
δ-Tocopherol (µg/g)	0.03	±	0.00	a	0.02	±	0.00	c	0.03	±	0.00	b
Total Tocopherol (µg/g)	9.97	±	0.05	a	8.66	±	0.06	b	7.49	±	0.05	c
α-Tocotrienol (µg/g)	17.03	±	0.42	b	20.06	±	0.90	a	18.84	±	0.23	ab
β-Tocotrienol (µg/g)	2.05	±	0.00	b	3.93	±	0.15	a	3.61	±	0.04	a
γ-Tocotrienol (µg/g)	9.06	±	0.20	a	9.05	±	0.46	a	5.63	±	0.03	b
δ-Tocotrienol (µg/g)	2.07	±	0.02	b	2.72	±	0.07	a	2.19	±	0.03	b
Total Tocotrienol (µg/g)	30.21	±	0.16	b	35.77	±	0.40	a	30.27	±	0.08	b
Total Tocols (µg/g)	40.18	±	0.81	ab	44.43	±	0.82	a	37.77	±	0.81	b

Results are presented as the mean ± standard error of the mean. Means within rows followed by different letters indicate significant differences; (Tukey–Kramer`s HSD for α = 0.05).

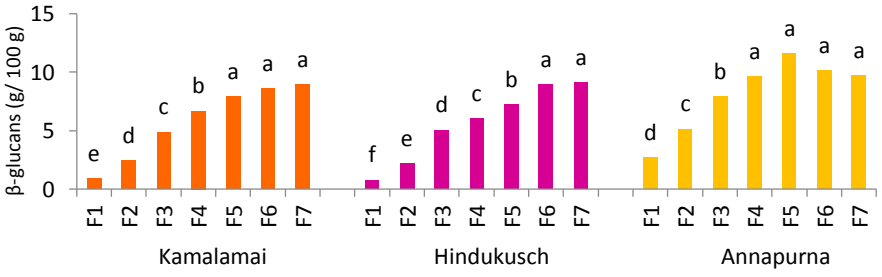


Figure IV.1. β-Glucan content (g/100 g) in barley pearling fractions. The results are presented as the mean; different letters indicate significant differences within the pearled fraction of each genotype; (Tukey-Kramer`s HSD for α = 0.05).

Arabinoxylans in cereals are mainly localized in the husks and cell walls of the outer layers of the grain including pericarp, testa and aleurone. In barley, aleurone cell walls are built up mainly of arabinoxylans (60 to 70%) whereas the endosperm cell walls contain only about 20 to 40% (Izydorczyk and Biliaderis, 2007). In our work, the arabinoxylan content of whole barley flours ranged from 5.5 to 6.6 g/100 g; Hindukusch being the highest (Table IV.2). These values were in accordance with those reported for different barley accessions in a previous work (Hassan et al., 2017). Contrary to the β-glucans, the arabinoxylans decreased progressively from the outer to the inner layers of the grain (Figure IV.2). The highest arabinoxylans amounts were observed in all F1 fractions. These mainly correspond to the hulls, testa and to the pericarp of both genotypes containing husks, and to pericarp, testa and some aleurone layers of Annapurna. The arabinoxylan level detected in the outermost layer F1 of the three genotypes was on average four times higher than that in whole barley flours. This finding may draw attention to barley husks as a good source of arabinoxylans.

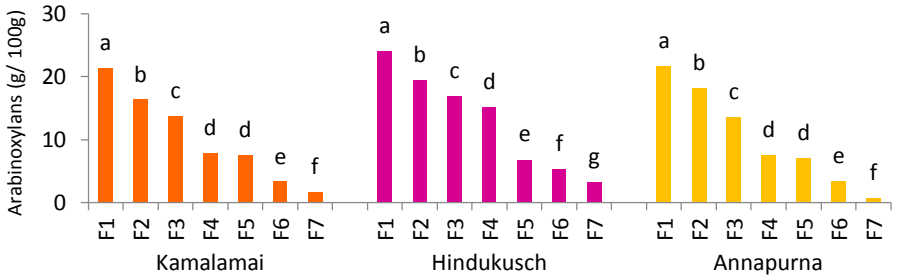


Figure IV.2. Arabinoxylan contents (g/100 g) in barley pearling fractions. Results are presented as the mean; different letters indicate significant differences within pearled fraction of each genotype; (Tukey–Kramer`s HSD for α = 0.05).

Tocols are plant metabolites with interest for their potential benefits for human health (Cavallero et al., 2004). Tocols, which consist of tocopherol and tocotrienols, are found in cereals at moderate levels ranging between 17 and 49 µg/g (Lampi et al., 2008; Badea et al., 2018). Among cereals, barley is one of the best sources of tocols due to the high content and favorable distribution of all eight major tocols, α-, β-, γ- and δ- tocopherols and tocotrienols (Moreau et al., 2007). While all tocol forms have similar antioxidant properties, α tocopherol (αT) is the only one that meets the Recommended Daily Allowance for vitamin E (Graebner et al., 2015; Idehen et al., 2017). In our study, there were detectable concentrations of the eight tocol isomers in all whole barley flours (Table IV.2). Total tocols ranged from 38 to 44 µg/g in good accordance with values found in other barley cultivars (Panfili et al., 2008; Badea et al., 2018; Martínez et al., 2018; Irakli et al., 2020). Tocotrienols accounted for 79% of the total tocols while the tocopherols were 21%. αT and α tocotrienols (αT3) were the main isomers in each tocol class, and their concentrations ranged from 6.2 to 8.3 µg/g and 17 to 20 µg/g respectively. Significant differences were identified among genotypes for most tocol forms. Both genotypes containing husks were the highest in αT, γT3 and total tocols. The tocopherol and tocotrienol contents in the fractions are shown in Figure IV.3A,B and Table IV.S.1. In line with previous finding, tocopherols and tocotrienols are distributed in a tissue specific manner with tocopherols mainly located in the germ whit tocotrienols in the aleurone and subaleurone layers (Panfili et al., 2008; Idehen et al., 2017; Badea et al., 2018). In this study, the highest tocopherol contents were detected between the middle fractions F3 to F6 of genotypes containing husk. This correspond to the highest amount of protein as described above, whereas the

content was uniformly distributed across the grain in the hull-less genotype Annapurna. Tocotrienols were more abundant in the outer layers F2 and F3 of the genotypes containing husks, and F1 to F3 of the hull-less one. Fractions F2 to F4 of the genotypes containing husks, and F1 to F3 of Annapurna would provide average tocol contents of 194 µg/g. These selected fractions contain over five times more tocols than whole flours and could be used as a valuable source of natural tocols.

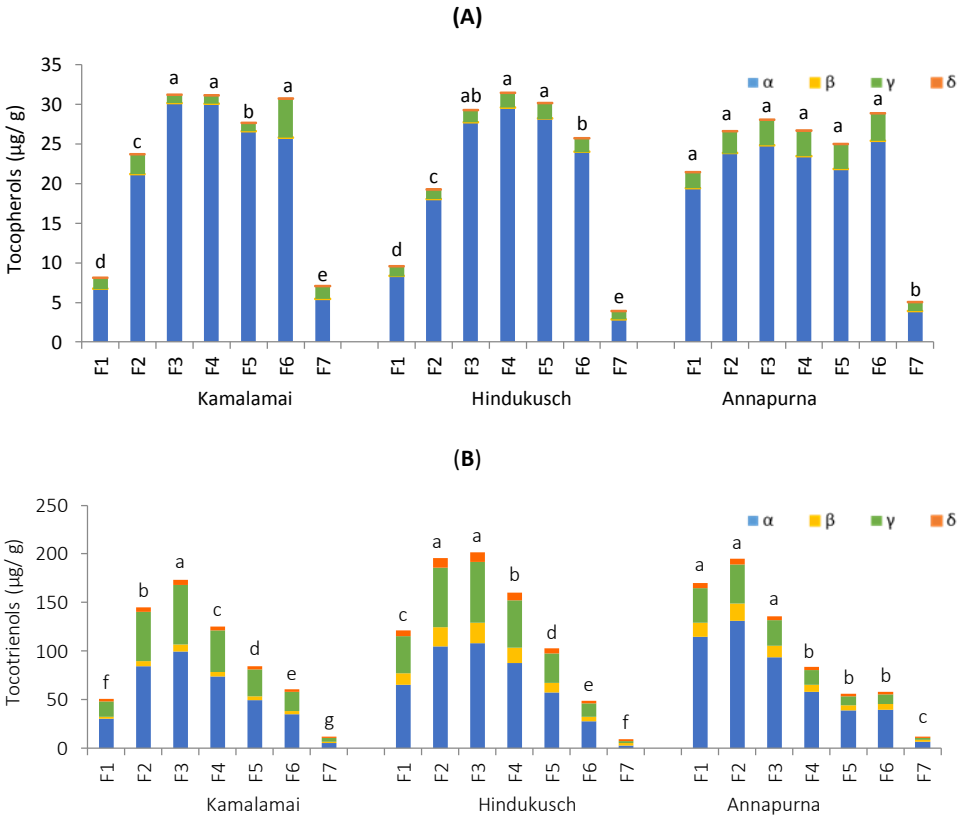


Figure IV.3. **(A)** α-, β-, γ-, δ- Tocopherol contents (µg/g), and **(B)** α-, β-, γ-, δ- Tocotrienol contents (µg/g) in barley pearling fractions. Results are presented as the mean; different letters indicate significant differences between pearled fractions of each genotype on log-transformed data; (Tukey-Kramer's HSD for α = 0.05).

IV.3.3. Phenolic Compounds (PCs) Contents

Phenolic compounds in barley can be found free or bound to the fiber being differentially distributed across the grain (Gamel and Abdel-Aal, 2012). The analysis of PCs in whole barley flours carried out by UPLC-MS/MS included free and bound forms (Table IV.3). Total free PCs ranged from 369 to 600 µg/g among which, flavan-3-ols accounted for 80%, phenolic acids for 16% and flavone glycosides for 4.1%. Total bound PCs ranged from 781 to 1194 µg/g and were comprised of 99.8% of phenolic acids and 0.2% of flavone glycosides. Bound phenolic acids were predominant in all whole flours representing 65-76% of the total PCs.

Table IV.3. Phenolic compounds contents (μg/g) in whole barley flours.

	Kamalamai				Hindukusch				Annapurna			
Flavan-3-ols	500.7	±	33.2	a	272.3	±	12.8	b	320.7	±	14.6	b
Free phenolic acids	81.1	±	6.1	a	70.6	±	2.3	ab	59.6	±	2.3	b
Free flavone glycosides	18.4	±	0.5	b	26.0	±	0.4	a	9.0	±	0.2	c
Total free	600.2	±	30.9	a	368.8	±	14.8	b	389.3	±	12.2	b
Bound phenolic acids	1092.1	±	113.4	ab	1192.6	±	215.6	a	779.5	±	32.9	b
Bound flavone glycosides	1.9	±	0.1	a	1.8	±	0.0	a	1.8	±	0.0	a
Total bound	1093.9	±	113.5	ab	1194.4	±	215.6	a	781.3	±	32.9	b
Total phenols	1694.1	±	117.4	a	1563.2	±	211.5	ab	1038.6	±	36.7	b

Results are presented as the mean ± standard error of the mean. Means within rows followed by different letters indicate significant differences; (Tukey–Kramer`s HSD for $\alpha = 0.05$).

Looking at the pearling fractions, the results showed a wide range of phenolic contents with differences between fractions and genotypes. Twenty-two different free phenols were detected. Their distribution within the fractions is detailed in Table IV.4. Procyanidin B3 and Prodelphinidin B4 were the most abundant flavan-3-ols in all the external fractions. Fractions F2 and F3 of the genotypes containing husks and F1 and F2 of Annapurna showed the highest concentrations of most flavan-3-ols which then progressively decreased until the endosperm except for catechins whose content did not vary as much between fractions. Fraction F1 was the richest in free phenolic acids in the three genotypes. This excludes gallic acid (the major free phenolic acid) whose content was similar within the external fractions. Some free phenolic acids, such as decarboxylated diferulic, hydroxybenzoic, caffeic and cinnamic, were exclusively detected in F1, F2 and F3 and were absent in the rest of the grain. Moreover, decarboxylated diferulic, hydroxybenzoic and 2,4-dihydroxybenzoic were not detected in Annapurna. F1 and F2 had also the highest concentrations of some free flavone glycosides such as apigenin 6-C-arabinoside 8-C-glucoside and ixovitexin 7-rutinoside, while the concentration of the remained free flavones was homogeneous or randomly distributed across the grain. Isoorientin was not detected in Annapurna nor was isovitexin 7-(6'''-sinapoylglucoside) 4'-glucoside in Hindukusch and Annapurna.

With reference to bound phenolic acids, their distribution across the grain was similar in the three genotypes (Table IV.5). The highest values were detected in the outermost fraction (F1) and gradually decreased toward the core of the grain, as did arabinoxylans, with which they are esterified. In fact, arabinoxylans positively correlated with bound phenolic acids ($r = 0.93$, $p < 0.001$). Ferulic acid was found to be the most abundant bound phenolic acid found in the three genotypes, accounting for 68–83% of total bound; p-coumaric acid came second in the genotypes containing husks (12-15%) and the diferulic acid in Annapurna (6%). Several authors detected high amounts of bound phenolic acids in husks (Izydorczyk and Biliaderis,2007; Izydorczyk et al., 2014). This might explain the results observed in our study: the genotypes containing husks being the richest in these compounds. In general, F1 was remarkably high in bound phenolic acids and might provide an important source of antioxidants. Minor bound phenolic acids, such as hydroxybenzoic acid, were uniformly distributed across the grain, and the distribution of others, like isoferulic, hydroxybenzoic, 2,4-dihydroxybenzoic, caffeic, sinapic and cinnamic acids, was not very marked. Bound diferulic tetrahydrofuran acid seemed to be exclusively present in the husks and pericarp layers as it was only detected in the F1 and F2 of both genotypes containing husks. Bound flavone glycosides showed similar distribution pattern as did the free forms.

Considering total PCs, similar distribution patterns between pearling fractions were observed in the three genotypes (Figure IV.4). F1 to F3 of the genotypes containing husks were those with the highest total PCs with the average content of 4339 µg/g, being on average 3 times higher than the contents in whole flours. On the contrary, F1 of the hull-less genotype was the highest in total PCs.

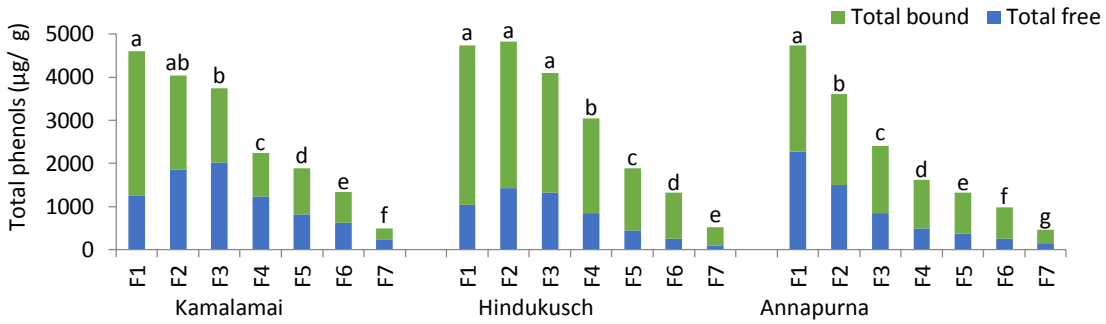


Figure IV.4. Total phenols contents (µg/g) in seven pearling fractions of three food barley genotypes. Results are presented as the mean; different letters indicate significant differences between pearled fractions of each genotype on log-transformed data; (Tukey–Kramer`s HSD for α = 0.05).

Most studies into the spatial distribution of PCs in barley have focused on either the total PCs by colorimetric methods (Marconi et al., 2000; Blandino et al., 2015) or just the major phenolic acids (Holtekjølen et al., 2011; Giordano et al., 2019), or have analyzed representative phenolic acids and free flavan-3-ols in a few fractions of previously dehulled cultivars (Gangopadhyay et al., 2018; Irakli et al., 2020). The results obtained in the present study show for the first time the specific distribution of a great variety of phenolic compounds determined by UPLC-MS/MS. These include phenolic acids, flavan-3-ols and flavone glycosides in seven pearling fractions in three food barley genotypes differing in the presence of the husk in the threshed grain.

Table IV.4. Free phenolic compounds contents (µg/g) in barley pearling fractions.

	Flavan-3-ols (µg/g)				**Phenolic acids** (µg/g)											**Flavone glycosides** (µg/g)						
	Cat	Cat-g	Pc B3	Pd B4	Gallic	Ferulic	DC Dif	*p*-Cm	*p*-OHB	OHB	2,4-diOHB	Vanill	Caff	Syring	Cinna	Ap g	Isosc g	Isosc r	Isoor	Isov g	Isov g-g	Isov r
												Kamalamai										
F1	53 d	102 b	374 c	312 c	35.9 a	27.1 a	1.1 a	25.7 a	21.5 a	10.9a	16.9 a	78.4 a	11.6 b	23.3 a	12.8 a	13.8 a	13.4 ab	8.3 a	7.3 a	0.2 d	1.7 a	1.8 a
F2	96 c	173 a	662 a	536 b	37.0 a	20.0 a	1.1 a	17.2 a	16.5 b	10.8a	14.5 b	48.1 b	12.8 a	17.2 b	11.5 b	10.0 b	14.7 a	8.1 a	7.1 ab	0.2 cd	1.7 a	1.1 ab
F3	125 ab	194 a	740 a	644 a	39.8 a	12.8 b	-	6.6 b	12.8 c	-	-	14.6 c	12.6 a	10.6 c	-	3.4 c	10.4 de	7.2 b	6.6 b	0.4 bc	0.3 b	0.7 bc
F4	143 a	125 b	434 b	322 c	38.8 a	7.2 c	-	3.5 bc	11.3 d	-	-	8.3 d	-	8.3 d	-	1.4 d	11.4 cd	7.1 b	6.7 ab	0.7 a	-	0.4 c
F5	121 ab	76 c	264 d	192 d	37.4 a	7.0 cd	-	3.1 bc	11.1 d	-	-	8.6 d	-	8.3 d	-	1.0 e	12.8 bc	7.0 b	6.9 ab	0.7 a	-	0.4 c
F6	104 bc	56 d	202 e	134 e	28.3 b	5.3 de	-	2.7 c	10.9 d	-	-	7.0 d	-	7.5 d	-	0.7 f	12.3 bc	6.9 b	7.0 ab	0.5 ab	-	0.4 c
F7	73 d	19 e	61 f	36 f	18.0 c	4.1 e	-	0.4 d	1.5 e	-	-	2.5 e	-	1.1 e	-	0.1 g	9.5 e	1.0 c	1.0 c	0.1 e	-	0.1 d
SED	*0.1*	*0.1*	*0.1*	*0.1*	*0.1*	*0.1*	*0.1*	*0.3*	*0.03*	*0.1*	*0.4*	*0.1*	*0.2*	*0.1*	*0.1*	*0.1*	*0.04*	*0.02*	*0.03*	*0.2*	*0.1*	*0.2*
												Hindukusch										
F1	34 c	53 b	227 b	385 b	24.1 ab	24.6 a	1.9 a	14.2 a	21.8 a	15.5	40.6 a	58.4 a	11.7 a	15.0 a	-	11.3 a	8.3 b	9.1 a	7.7 a	1.5 a	-	2.2 a
F2	48 ab	100 a	359 a	612 a	27.8 ab	14.3 b	1.1 b	5.9 b	18.8 b	-	31.3 b	44.7 b	10.8 b	11.4 b	-	7.2 b	8.4 b	9.1 a	7.4 ab	2.1 a	-	2.0 ab
F3	58 a	101 a	349 a	544 a	29.5 ab	10.2 c	0.7 c	3.7 bc	16.5 c	-	21.7 c	36.2 c	10.5 b	9.4 c	-	4.3 c	8.3 b	8.0 b	7.0 bc	1.5 a	-	1.3 bc
F4	46 b	56 b	235 b	316 c	31.1 a	7.8 d	-	3.0 cd	15.3 d	-	15.9 d	28.8 d	-	8.4 cd	-	2.1 d	7.5 c	7.5 bc	6.9 c	0.8 b	-	0.9 cd
F5	33 c	24 c	107 c	129 d	32.6 a	6.2 d	-	1.9 de	14.1 e	-	14.1 d	23.5 e	-	7.9 d	-	1.2 e	8.7 b	7.3 c	6.9 c	0.6 bc	-	0.7 d
F6	27 c	14 d	49 d	48 e	20.5 bc	4.4 e	-	1.1 e	13.1 e	-	1.9 e	17.2 f	-	7.6 d	-	0.6 f	9.5 a	7.1 c	6.9 c	0.3 c	-	0.3 e
F7	16 d	2 d	13 e	8 f	16.1 c	3.5 f	-	-	10.5 f	-	-	5.9 g	-	0.1 e	-	0.1 g	8.3 b	1.0 d	1.0 d	0.1 d	-	0.03 f
SED	*0.1*	*0.1*	*0.03*	*0.1*	*0.1*	*0.1*	*0.1*	*0.2*	*0.02*	*0.1*	*0.1*	*0.1*	*0.2*	*0.04*		*0.1*	*0.03*	*0.02*	*0.02*	*0.2*		*0.2*
												Annapurna										
F1	91 b	349 a	927 a	554 a	25.4 a	11.9 a	-	4.5 a	12.4 a	-	-	25.8 a	11.3 a	11.7 a	12.1 a	3.7 a	10.7 a	8.5 a	-	0.2 bc	-	1.0 a
F2	85 c	245 a	601 b	339 ab	24.1 a	8.1 b	-	2.2 b	10.9 b	-	-	16.9 ab	10.3 b	9.2 b	11.4 ab	1.4 b	8.7 d	7.2 b	-	0.3 ab	-	0.5 b
F3	91 bc	139 b	302 c	164 b	22.3 a	6.1 bc	-	1.8 bc	10.9 b	-	-	12.5 b	-	7.9 c	10.9 b	0.5 c	9.0 cd	7.0 b	-	0.3 ab	-	0.2 c
F4	105 a	79 c	156 d	71 c	21.3 a	4.7 cde	-	1.8 bc	10.7 b	-	-	6.5 c	-	7.3 c	-	0.4 cd	9.7 bc	-	-	0.4 ab	-	0.2 c
F5	90 bc	53 c	108 e	49 c	21.0 a	5.2 cd	-	1.6 c	10.2 bc	-	-	6.4 c	-	7.1 c	-	0.4 cd	11.3 a	-	-	0.5 a	-	0.2 c
F6	89 bc	29 d	55 f	20 d	22.2 ab	3.6 e	-	-	10.3 bc	-	-	4.3 c	-	7.1 c	-	0.2 d	10.6 ab	-	-	0.1 c	-	-
F7	51 d	15 e	30 g	15 d	16.4 b	4.2 de	-	-	9.8 c	-	-	2.2 d	-	1.0 d	-	-	7.7 e	-	-	-	-	-
SED	*0.1*	*0.1*	*0.1*	*0.2*	*0.1*	*0.1*		*0.1*	*0.02*			*0.1*	*0.1*	*0.03*	*0.3*	*0.2*	*0.03*	*0.01*		*0.2*		*0.1*

Results are presented as the mean. Means within a column followed by different letters indicate significant differences on log-transformed data; (Tukey-Kramer's HSD for $\alpha = 0.05$). SED: standard error of the difference between means. Cat: Catechin, Cat-g: Catechin-glucoside, Pc B3: Procyanidin B3, Pd B4: Prodelphinidin B4, DC Dif: Decarboxylated diferulic acid, *p*-Cm: *p*-coumaric acid, *p*-OHB: *p*-hydroxybenzoic acid, OHB: *m*- or *o*-hydroxybenzoic acid, 2,4-diOHB: 2,4-dihydroxyenzoic acid, Vanill: Vanillic acid, Caff: Caffeic acid, Syring: Syringic acid, Cinna: Cinnamic acid, Ag g: Apigenin 6-C-arabinoside 8-C-glucoside, Isosc g: Isoscoparin 7-glucoside, Isosc r: Isoscoparin 7-rutinoside, Isoor: Isoorientin, Isov g: Isovitexin 7-glucoside, Isov g-g: Isovitexin 7-(6'''-sinapoylglucoside) 4'-glucoside, Isov r: Isovitexin 7-rutinoside. - Not detected

Table IV.5. Bound phenolic compounds contents (µg/g) in barley pearling fractions.

	Phenolic acids (µg/g)																**Flavone glycosides** (µg/g)			
	Ferulic	isoF	DiF	TriF	DC DiF	DiF THF	*p*-Cm	*m*-Cm	*p*-OHB	OHB	2,4-diOHB	Vanillic	Caffeic	Syringic	Sinapic	Cinna	Ag g	Isosc g	Isosc r	Isov g
										Kamalamai										
F1	1367 a	17 bc	330 a	21.2 a	34.3 a	5.5 a	978 a	21.3 a	22.8 a	3.0 c	3.5 bc	117 a	5.1 c	19.6 a	14.1 ab	3.8 d	1.6 a	1.7 d	-	0.05 b
F2	1260 a	15 bc	165 b	15.8 a	18.2 b	1.9 b	523 b	8.1 b	15.3 b	4.6 ab	4.2 b	78 b	7.2 b	15.9 a	20.3 a	5.5 a	1.1 a	2.4 a	-	0.09 a
F3	1184 a	15 bc	151 b	31.7 a	3.0 c	-	182 c	3.5 c	11.1 c	4.9 ab	6.0 a	44 c	16.1 a	9.4 b	21.1 a	5.9 a	0.2 b	2.1 b	-	-
F4	804 b	20 b	69 c	3.8 b	1.1 d	-	65 d	2.2 cd	6.8 d	4.8 ab	2.8 c	18 d	3.9 cd	4.9 c	11.2 bc	4.5 b	0.1 c	2.0 bc	-	-
F5	833 b	32 a	70 c	2.2 b	0.9 de	-	53 de	3.4 c	6.6 d	5.4 a	-	17 d	3.8 cd	4.4 c	10.0 bc	4.4 bc	0.1 c	2.1 bc	-	-
F6	575 c	14 c	46 d	2.5 b	0.7 e	-	38 e	1.6 d	5.1 e	4.2 b	-	11 e	2.8 d	3.3 d	7.1 c	3.9 cd	0.1 c	1.9 c	-	-
F7	206 d	6 d	17 e	1.8 b	0.1 f	-	14 f	0.9 e	3.3 f	4.3 b	-	4 f	0.4 e	2.1 e	1.1 d	3.1 e	0.01 d	0.3 e	-	-
SED	*0.1*	*0.1*	*0.1*	*0.3*	*0.1*	*0.1*	*0.1*	*0.2*	*0.1*	*0.3*	*0.3*	*0.1*	*0.1*	*0.1*	*0.2*	*0.04*	*0.3*	*0.03*		*0.01*
										Hindukusch										
F1	2030 a	16 bc	342 a	29.5 a	18.7 a	1.9 a	939 a	12.1 a	45.8 a	4.7 a	37.3 ab	128 a	5.2 bc	14.7 a	44.1 ab	5.3 b	0.8 a	2.0 a	1.7 a	0.2 a
F2	2237 a	20 ab	374 a	26.2 a	9.2 b	0.6 b	355 b	3.7 b	48.0 a	5.3 a	77.5 a	135 a	6.8 ab	11.5 ab	57.4 a	6.3 a	0.3 b	2.0 ab	1.7 a	0.1 b
F3	1968 a	25 ab	309 a	23.1 a	5.8 b	-	154 c	1.7 c	33.3 b	5.3 a	63.9 a	99 ab	8.7 a	10.3 bc	47.9 ab	5.6 ab	0.2 b	1.8 bc	1.6 a	0.1 b
F4	1643 a	30 a	204 b	10.1 b	3.0 c	-	111 c	1.5 c	22.5 c	5.7 a	26.3 b	69 b	4.4 c	8.4 c	32.1 b	5.0 b	0.1 c	1.7 c	1.6 a	0.1 b
F5	1130 b	24 ab	114 c	6.7 bc	1.9 cd	-	53 d	1.1 c	13.8 d	5.6 a	7.4 c	41 c	3.1 d	5.7 d	17.1 c	4.2 c	0.1 c	1.8 bc	1.1 a	0.1 b
F6	840 b	25 ab	74 d	4.2 c	1.3 d	-	35 d	1.3 c	10.1 e	5.3 a	4.2 c	29 c	2.8 d	4.4 d	12.2 c	3.7 c	0.1 c	1.9 abc	1.6 a	0.04 c
F7	353 c	10 c	24 e	1.2 c	0.2 e	-	9 e	0.1 d	5.3 f	5.4 a	3.3 c	9 d	0.4 e	2.4 e	1.1 d	3.2 d	-	0.3 d	-	-
SED	*0.1*	*0.2*	*0.1*	*0.2*	*0.7*	*0.1*	*0.2*	*0.2*	*0.1*	*0.4*	*0.2*	*0.1*	*0.1*	*0.1*	*0.2*	*0.04*	*0.2*	*0.03*	*0.01*	*0.2*
										Annapurna										
F1	1850 a	15 bc	262 a	28.5 a	5.2 a	0.30	169 a	1.7 a	11.1 a	4.6 ab	-	43 a	5.9 ab	10.2 a	42.0 a	6.3 a	0.2 a	2.3 a	-	-
F2	1697 a	16 bc	179 b	13.3 b	1.6 b	-	78 b	0.8 b	8.7 b	4.9 a	-	26 b	11.7 a	7.2 b	27.9 b	5.1 b	0.1 b	2.2 ab	-	-
F3	1323 b	19 ab	109 c	3.8 c	0.9 c	-	37 c	0.6 bc	6.7 c	4.6 ab	-	16 c	3.6 b	5.2 c	15.4 c	4.4 b	-	2.1 bc	-	-
F4	968 c	25 a	69 d	3.1 cd	1.0 c	-	21 d	0.5 bc	4.9 d	4.3 bc	-	9 d	2.8 b	3.5 d	8.3 d	3.5 c	-	2.0 cd	-	-
F5	809 d	20 ab	58 d	2.1 de	2.3 b	-	20 d	0.5 bc	4.1 e	4.0 c	-	7 de	2.7 b	2.8 e	5.4 e	3.4 cd	-	1.9 d	-	-
F6	623 e	14 c	42 e	2.3 d	2.1 b	-	15 d	0.5 bc	3.9 e	4.3 bc	-	6 e	2.6 b	2.5 e	4.1 e	3.3 cd	-	1.9 d	-	-
F7	259 f	6 d	18 f	1.4 e	0.1 d	-	7 e	0.4 c	3.1 f	4.1 c	-	3 f	-	1.9 f	-	2.9 d	-	-	-	-
SED	*0.1*	*0.1*	*0.1*	*0.1*	*0.1*	*0.0*	*0.1*	*0.2*	*0.04*	*0.1*		*0.1*	*0.3*	*0.1*	*0.1*	*0.1*	*0.1*	*0.02*		

Results are presented as the mean. Means within a column followed by different letters indicate significant differences on log-transformed data; (Tukey-Kramer's HSD for α= 0.05). SED: standard error of the difference between means. iso-F: isoferulic, DiF: Diferulic acid, TriF: Triferulic acid, DC DiF: Decarboxylated diferulic acid, DiF THF: Diferulic tetrahydrofuran, *p*-Cm: *p*-Coumaric acid, *m*-Cm: *m*-Coumaric acid, *p*-OHB: *p*-hydroxybenzoic acid, OHB: *m*- or *o*-hydroxybenzoic acid, 2,4diOHB: 2,4-dihydroxyenzoic acid, Cinna: Cinnamic acid, Ag-g: Apigenin 6-C-arabinoside 8-C-glucoside, Isosc g: Isoscoparin 7-glucoside, Isosc r: Isoscoparin 7-rutinoside, Isov g: Isovitexin 7-glucoside. - Not detected

IV.3.4. Anthocyanins

In this work, the anthocyanin content of Kamalamai and Annapurna was negligible, whereas a total of 24 anthocyanins were detected in the purple Hindukusch genotype. These included pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvinidin conjugates of glucose, acetylglucose, malonylglucose, dimalonylglucose, arabinose, rutinose and dihexose. The total anthocyanin content was 47 µg/g, which was lower than or similar to those detected in other purple barley cultivars (Lee et al., 2013; Zhang et al., 2017; Martínez et al., 2018). This indicates a great diversity, probably associated with genetic and environmental factors. Cyanidin dimalonyl glucoside represented 45% of the total anthocyanins followed by cyaniding glucoside, which accounted for 35%. Several studies have shown that anthocyanins are mainly concentrated in the pericarp and some aleurone layers that provide the grain colour (Idehen et al., 2017). In this study, the distribution of anthocyanins between the barley fractions showed fractions F1 and F2 as the highest (Tables IV.6 and IV.S.2). The total anthocyanin contents detected in F2 was upon 10 times higher than that in whole flour. Like most phenolic compounds, the anthocyanin concentration decreased progressively from F2 to F7 where minor contents were detected.

Table IV.6. Anthocyanins contents (µg/g) in barley pearling fractions

	Pelagonidins	Cyanidins	Peonidins	Delphinidins	Petunidins	Malvinidins	Total Anthocyanins
			Hindukusch				
F1	41.0 a	285.3 a	5.87 b	17.8 a	3.42 a	1.15 a	354.5 a
F2	50.0 a	366.3 a	21.59 a	20.3 a	2.01 a	1.29 a	461.2 a
F3	25.0 b	188.6 b	0.45 bc	12.6 b	1.50 ab	1.06 a	229.2 b
F4	9.6 c	72.5 c	0.25 bc	6.4 c	0.89 ab	0.61 ab	90.2 c
F5	4.6 d	32.8 d	0.17 bcd	3.4 d	0.59 bc	0.40 abc	42.0 d
F6	2.7 e	19.3 e	0.10 cd	2.1 e	0.20 c	0.30 bc	24.7 e
F7	1.1 f	7.0 f	0.02 d	0.7 f	0.04 d	0.14 c	9.0 f
SED	*0.1*	*0.1*	*0.7*	*0.1*	*0.3*	*0.3*	*0.1*

Results are presented as the mean. Means within a column followed by different letters indicate significant differences on log-transformed data; (Tukey-Kramer's HSD for α= 0.05). SED: standard error of the difference between means.

IV.3.5. Antioxidant Capacity (AC)

Antioxidant capacity is an important integrative parameter for evaluating the potential health benefits of foods. The oxygen radical absorbance capacity (ORAC) values of the free, bound and total phenolic compounds of whole barley flours were in the ranges from 46 to 71, 28 to 58, and 74 to 119 µmol Trolox/g respectively (Table IV.7).

Table IV.7. Antioxidant Capacity (µmol Trolox/g) of whole barley flours.

	Kamalamai	**Hindukusch**	**Annapurna**
Free AC	70.7 ± 1.3 a	61.9 ± 0.2 b	45.6 ± 0.9 c
Bound AC	39.5 ± 2.4 b	57.4 ± 3.3 a	28.1 ± 0.9 c
Total AC	110.2 ± 1.1 a	119.3 ± 3.5 a	74.0 ± 1.8 b

Results are presented as the mean ± standard error of the mean. Means within a rows followed by different letters indicate significant differences; (Tukey-Kramer's HSD for α= 0.05).

The contribution of free PCs to the total AC was higher than that of the bound PCs. This suggested that free PCs had excellent AC as determined by the ORAC assay, since they were at much lower

concentrations than bound PCs as explained above. The average ORAC values of genotypes containing husks were significantly higher than that of Annapurna. The ORAC values detected in the three barley genotypes were similar or relatively higher than those reported for whole barley flours in previous works (Madhujith and Shahidi, 2009; Yoshida et al., 2010; Zhu et al., 2015; Xia et al., 2019).

When the ORAC assay was measured in the pearling fractions, the highest values were found in the first three fractions of the genotypes containing husks and F1 of Annapurna (Figure IV.5). F1 of Kamalamai showed a similar AC to F2 and F3 despite having higher amounts of total PCs. These results should be attributed to the strong antioxidant capacity of free PCs in F2 and F3, which matched the ORAC values of F1. In the case of the purple Hindukusch genotype, the highest AC was detected in F2. This fraction contained the highest amount of free PCs, including anthocyanins, whose AC exceeded that of the components in F1 and F3. Finally, F1 of Annapurna (equivalent to F2 of the genotypes containing husks) showed the highest AC, and this value decreased going toward the inner part of the grain as did free, bound and total PCs. Based on these results, the 0-15% outer fractions of the genotypes containing husks and the 0-5% fraction of Annapurna made significant contributions to the total antioxidant capacity of all whole flours.

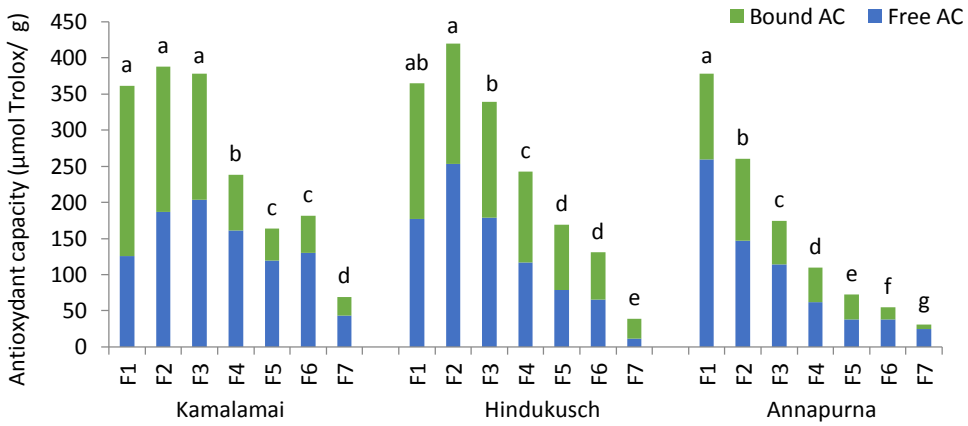


Figure IV.5. Antioxidant capacity (µmol Trolox/g) detected in seven pearling fractions in three food barley genotypes. Results are presented as the mean; different letters indicate significant differences between pearled fractions of each genotype on log-transformed data; (Tukey–Kramer`s HSD for α = 0.05).

IV.3.6. Association between Variables

Figure IV.6 shows the PCA biplot of ash, protein, bioactive compounds and antioxidant capacity in the seven pearling fractions of the three food barley genotypes. The first two principal component axes explained more than two thirds of the total variability in the standardized data from Figures IV.1-IV.3 and IV.5 and Tables IV.4 and IV.5. The first axis, explaining 50% of the total variation, seemed to be related to the contrast between the bioactive compounds found in the outer fractions (ash, arabinoxylans, phenolic compounds and tocotrienols) and the β-glucans present in the endosperm. The second, explaining 17% of the total, seemed to be related to compounds such as tocopherols, protein and minor phenolic compounds like catechins (Cat) and bound hydroxybenzoic acid (OHB) found in intermediate layers.

The size of the circles for each pearling fractions was proportional to their total antioxidant capacity and clearly descends from fraction F1 to F7 for all genotypes. The distribution pattern was similar for all genotypes, with little differences for the outermost fractions. The size of the blue squares representing the 28 phenolic compounds was proportional to their content within the fractions. Overall, Hindukusch, with the outermost fractions being further away from the origin of coordinates, seemed higher in such bioactive compounds as tocotrienols, prodelphinidin B4 (PdB4) bound ferulic acid (B-F), and anthocyanins (AN) than the other two genotypes. Highlighted in quadrant II, bound p-coumaric acid (p-Cm) showed higher content in the fractions that mainly contain husks, and in quadrant I the bound ferulic (B-F) and diferulic (B-DiF) acids, and the procyanidin B3 (PcB3) and prodelphinidin B4 (PdB4) present in the outer external fraction.

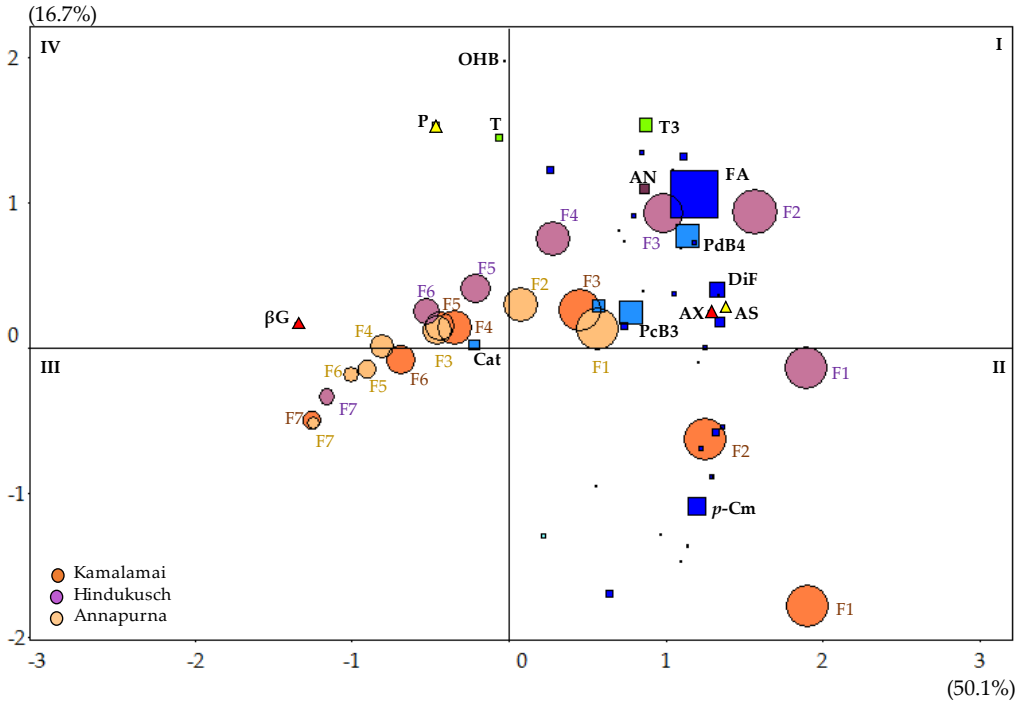


Figure IV.6. Biplot of the Principal Component Analysis of ash, protein, dietary fiber and 60 bioactive compounds in seven sequential pearling fractions in three food barley genotypes. The size of the squares is proportional to each phenolic compound content; Circle size is proportional to total antioxidant capacity. F: fraction; AS: Ash; P: Protein; βG: β-glucans; AX: arabinoxylans; T: tocopherols; T3: tocotrienols. The following eight main phenolic compounds are also labeled: FA: bound ferulic acid; p-Cm: bound p-coumaric acid; DiF: bound diferulic acid; Cat: catechins; OHB: m- or o-hydroxybenzoic acid; PcB3: procyanindin B3, PdB4: prodelfinidin B4; Light blue squares: flavone glycosides; AN: total anthocyanins.

Whereas PCA is an extremely powerful tool for the visualization of multidimensional data, it does not allow for statistical inferences about contents across grain sections. These comparisons are shown in Figure IV.7, which summarizes the Tukey's HSD Mean Comparison groupings for four sequential spatial sections of the barley grain, determined by differential aggregation of the seven pearling fraction from each of the three, hulled, partially-hull-less and hull-less food barley genotypes used. The husks had a high content in ash, arabinoxylans, some specific major phenolic compounds such as bound p-coumaric, ferulic and diferulic acids, procyanidin B3 and prodelphinidin

B4, resulting in a high antioxidant capacity, similar to that of the outermost layers. Current regulations require removal of the inedible husks from hulled barley to be used for human food (FDA, 2006). However, due to its high content of bioactive compounds, either the whole hulled barley grains or the external husks of food hulled barley genotypes should be considered as a valuable material for animal feeds. Apart from providing the same concentration of bioactive compounds as husks, the outermost layers also had high contents of tocols. Therefore, the outermost layers of naked barley or previously de-husked barley for human food represent the most valuable source of bioactive compounds. The intermediate layers, provide high contents of protein, β-glucans, tocopherols and some phenolic compounds such as catechins and hydroxybenzoic acid. Finally, the endosperm has the highest β-glucans contents and relative high presence of catechins and hydroxybenzoic acid.

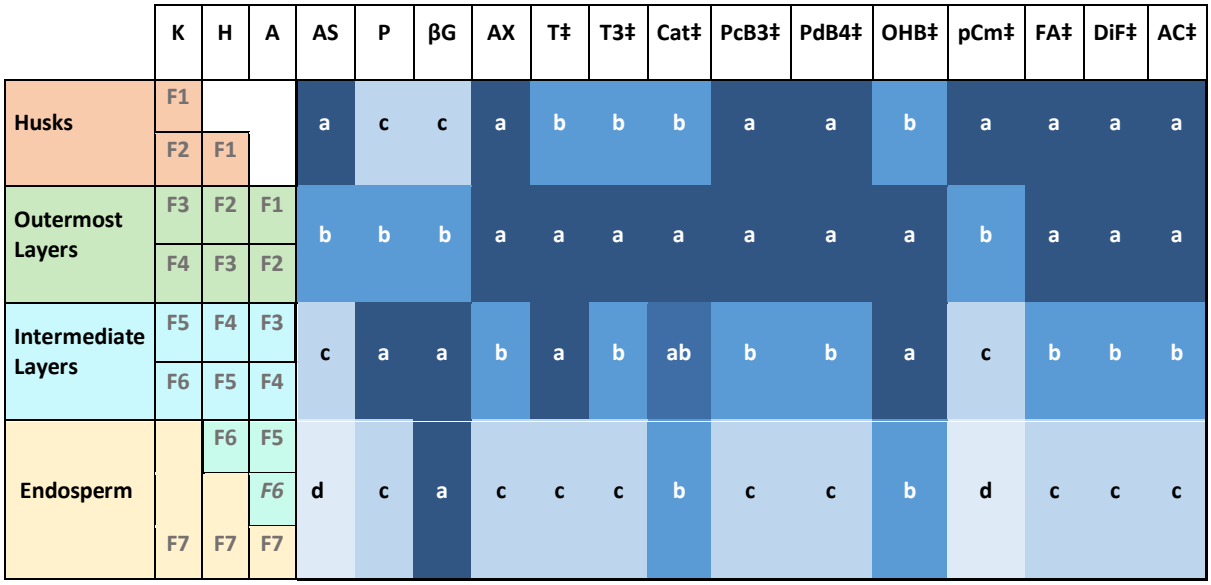


Figure IV.7. Tukey's HSD groupings for a number of compounds for the different sections of the grains across three food barley genotypes. The colored left columns represent the different pearling fractions (F1-F7) of the Kamalamai (K), Hindukusch (H) and Annapurna (A) used for the aggregation into four grain sections shown in the first column. A: Ash; P: protein; βG: β-glucans; AX: arabinoxylans; T: tocopherols; T3: tocotrienols; Cat: catechins; PcB3: Procyanidin B3; PdB4: prodelphinidin B4; OHB: m- or o-hydroxybenzoic acid; pCm: bound p-coumaric acid, FA: bound ferulic acid; DiF: bound diferulic acid; AC: total antioxidant capacity. ‡ Analyses carried out on log-transformed data.

In conclusion, high β-glucan food barley genotypes can be an excellent source of not just of dietary fiber but a plethora of phenolic compounds with potential health promoting properties. Whole or lightly pearled grains, as well as their specific pearling fractions, could be used as a diverse source of valuable functional ingredients.

IV.4. Author Contributions

Conceptualization, M.M. and M.P.R.; methodology, M.M.-S., M.M. and M.P.R.; formal chemical analyses, M.M.-S., A.M. and E.P.; statistical analyses and data curation, M.M.-S. and I.R.; writing—original draft preparation, M.M.-S.; writing—review and editing, I.R., M.P.R. and M.M.; supervision,

M.M.; funding acquisition, I.R. and M.M. All authors have read and agreed to the published version of the manuscript.

IV.5. Funding

This work was funded by project AGL 2015-69435-C3-1 from the Spanish Ministry of Economy and Competitiveness. Mariona Martínez-Subirà was supported by a pre-doctoral fellowship (BES-2016-078654/AGL 2015-69435-C3-1).

IV.6. Acknowledgments

Our thanks to Semillas Batlle, SA (Bell-lloc d'Urgell, Lleida, Spain) for providing the food barley genotypes used in this work.

IV.7. Conflicts of Interest

The authors declare no conflict of interest.

IV.8. Supplementary information

Table IV.S.1. Tocopherol and tocotrienol contents pearling fraction of three barley genotypes.

	Tocopherols (T) (μg/g)										Tocotrienols (T3) (μg/g)									
	α		β		γ		δ		Total T		α		β		γ		δ		Total T3	
Kamalamai																				
F1	6.7	d	0.036	ab	1.37	d	0.028	c	8.1	d	30.4	f	2.24	f	15.8	f	2.49	e	50.9	f
F2	21.2	c	0.036	ab	2.40	b	0.050	b	23.7	c	84.1	b	5.52	b	50.8	b	4.70	a	145.1	b
F3	30.1	a	0.041	a	1.01	e	0.081	a	31.2	a	99.8	a	6.65	a	61.5	a	5.37	a	173.3	a
F4	30.0	a	0.038	ab	1.02	e	0.081	a	31.1	a	73.6	c	4.90	c	42.4	c	4.04	b	125.0	c
F5	26.6	b	0.034	b	1.01	e	0.078	a	27.7	b	49.7	d	3.47	d	27.7	d	3.29	c	84.2	d
F6	25.8	b	0.042	a	4.91	a	0.079	a	30.8	a	35.3	e	2.77	e	19.8	e	2.77	d	60.6	e
F7	5.4	e	0.036	ab	1.53	c	0.028	c	7.0	e	5.3	g	1.68	g	3.6	g	1.66	e	12.2	g
SED	*0.01*		*0.043*		*0.02*		*0.026*		*0.01*		*0.02*		*0.02*		*0.02*		*0.04*		*0.02*	
Hindukusch																				
F1	8.3	d	0.027	cd	1.19	d	0.026	ab	9.5	d	65.1	c	12.23	c	38.1	c	6.02	c	121.4	c
F2	18.0	c	0.026	d	1.17	d	0.024	ab	19.2	c	104.6	a	19.95	a	61.4	a	9.62	a	195.5	a
F3	27.7	a	0.033	ab	1.45	c	0.024	ab	29.2	ab	108.3	a	20.70	a	62.6	a	10.13	a	201.7	a
F4	29.5	a	0.035	a	1.81	ab	0.026	ab	31.4	a	87.9	b	15.90	b	48.2	b	7.92	b	159.9	b
F5	28.1	a	0.036	a	1.96	a	0.025	ab	30.2	a	57.6	c	9.89	d	30.0	d	5.13	d	102.7	d
F6	23.9	b	0.029	bcd	1.67	b	0.033	a	25.7	b	27.7	d	4.91	e	13.2	e	3.13	e	49.0	e
F7	2.8	e	0.030	bc	1.03	e	0.016	b	3.9	e	3.1	e	2.12	f	2.2	f	1.66	f	9.1	f
SED	*0.03*		*0.033*		*0.03*		*0.136*		*0.03*		*0.03*		*0.03*		*0.03*		*0.03*		*0.03*	
Annapurna																				
F1	19.4	a	0.040	a	2.03	b	0.048	a	21.5	a	114.5	a	14.81	a	35.6	a	5.07	ab	170.0	a
F2	23.8	a	0.037	a	2.75	ab	0.055	a	26.6	a	131.2	a	17.92	a	40.1	a	5.73	a	195.0	a
F3	24.8	a	0.042	a	3.20	a	0.058	a	28.1	a	93.4	a	12.28	ab	25.9	a	4.26	b	135.9	a
F4	23.4	a	0.040	a	3.25	a	0.057	a	26.7	a	58.1	b	7.52	bc	14.9	b	3.13	c	83.7	b
F5	21.8	a	0.041	a	3.12	ab	0.058	a	25.0	a	38.8	b	5.20	c	9.6	b	2.57	c	56.2	b
F6	25.3	a	0.046	a	3.46	a	0.061	a	28.3	a	39.2	b	5.78	c	9.4	b	2.55	c	57.0	b
F7	3.8	b	0.038	a	1.16	c	0.021	b	5.1	b	6.3	c	2.18	d	2.1	c	1.61	d	12.2	c
SED	*0.11*		*0.060*		*0.11*		*0.088*		*0.11*		*0.11*		*0.13*		*0.13*		*0.06*		*0.11*	

Results are presented as the mean. Means within a column followed by different letters indicate significant differences on log-transformed data; (Tukey-Kramer HSD for α= 0.05). SED: standard error of the difference between means.

Table IV.S.2. Anthocyanin contents (µg/ g) in the pearling fractions of the partially hull-less and purple genotype.

	F1		F2		F3		F4		F5		F6		F7		*SED*
Pelargonidin glucoside	4.55	a	5.48	a	2.77	b	1.14	c	0.58	d	0.31	e	0.11	e	*0.08*
Pelargonidin acetylglucoside	0.10	a	0.12	a	0.08	a	0.03	b							*0.12*
Carboxypyrano pelargonidin glu	0.25	ab	0.28	a	0.15	b	0.08	c	0.04	d	0.03	d	0.01	e	*0.17*
Pelargonidin malonylglucoside	23.18	a	28.40	a	14.68	b	5.55	c	2.59	d	1.58	e	0.59	f	*0.10*
Pelargonidin dimalonylglucoside	12.63	a	15.37	a	7.35	b	2.83	c	1.38	d	0.80	e	0.35	f	*0.13*
Cyanidin arabinoside	0.19	a	0.24	a	0.14	b	0.05	c	0.03	d					*0.09*
Cyanidin glucoside	94.90	a	121.81	a	66.00	b	25.64	c	11.69	d	7.07	e	2.30	f	*0.08*
Cyanidin acetylglucoside	2.04	a	2.42	a	1.36	b	0.53	c	0.25	d	0.17	e	0.04	f	*0.10*
Cyanidin malonylglucoside	0.89	a	0.64	a	0.39	b	0.22	c	0.14	c	0.08	d	0.01	e	*0.13*
Cyanidin dimalonylglucoside	186.08	a	241.24	a	120.71	b	46.04	c	20.68	d	12.00	e	4.70	f	*0.09*
Peonidin acetylglucoside	0.07	b	0.10	b	0.20	a									*0.14*
Peonidin malonylglucoside	0.28	a	0.28	a	0.18	a	0.18	a	0.15	a	0.07	b	0.01	c	*0.20*
Peonidin dimalonylglucoside	5.52	b	21.21	a	0.07	bc	0.07	bc	0.03	bc	0.02	bc	0.01	c	*0.99*
Delphinidin arabinoside							0.05	a	0.03	a					*0.16*
Delphinidin glucoside	1.91	a	2.94	a	2.15	a	1.49	a	0.53	b	0.26	b	0.08	c	*0.23*
Delphinidin malonylglucoside	15.33	a	16.80	a	10.04	b	4.54	c	2.70	d	1.78	e	0.65	f	*0.09*
Delphinidin dimalonylglucoside	0.59	a	0.57	a	0.39	ab	0.28	b	0.14	c	0.08	d	0.01	e	*0.13*
Petunidin glucoside	0.44	a	0.46	a	0.40	a	0.23	b	0.17	b					*0.05*
Petunidin malonylglucoside	2.49	a	1.06	a	0.74	a	0.43	ab	0.26	ab	0.13	b	0.01	c	*0.47*
Petunidin rutinoside	0.15	ab	0.19	a	0.18	a	0.14	ab	0.09	bc	0.07	c	0.02	d	*0.17*
Petunidin hexoside hexoside	0.22	a	0.17	ab	0.09	bc	0.05	cd	0.04	d					*0.20*
Petunidin dimalonylglucoside	0.13	a	0.12	a	0.09	a	0.04	b	0.03	b					*0.01*
Malvidin malonylglucoside	1.01	a	1.15	a	1.00	a	0.57	a	0.39	ab	0.28	ab	0.12	b	*0.44*
Malvidin hexoside hexoside	0.03	ab	0.05	a	0.06	a	0.04	ab	0.02	bc	0.02	bc	0.01	c	*0.21*

Results are presented as mean. Means within a rows followed by different letters indicate significant differences on log-transformed data; (Tukey-Kramer HSD for α= 0.05). SED: standard error of the difference between means.

IV.9. References

Abdel-Aal E-SM, Choo T-M, Dhillon S, Rabalski I (2012) Free and Bound Phenolic Acids and Total Phenolics in Black, Blue, and Yellow Barley and Their Contribution to Free Radical Scavenging Capacity. Cereal Chem. J. 89:198–204.

Andersson AAM, Lampi A-M, Nyström L, Piironen V, Li L, Ward JL, Gebruers K, Courtin CM, Delcour JA, Boros D, Fraś A, Dynkowska W, Rakszegi M, Bedo Z, Shewry PR, Aman P (2008) Phytochemical and Dietary Fiber Components in Barley Varieties in the Healthgrain Diversity Screen. J. Agric. Food Chem. 56: 9767–9776.

Badea A, Carter A, Legge WG, Swallow K, Johnston SP, Izydorczyk MS (2018) Tocols and oil content in whole grain, brewer's spent grain, and pearling fractions of malting, feed, and food barley genotypes. Cereal Chem. J. 95: 779–789.

Bai J, Ren Y, Li Y, Fan M, Qian H, Wang L, Wu G, Zhang H, Qi X, Xu M, Rao Z (2019) Physiological functionalities and mechanisms of β-glucans. Trends Food Sci. Technol. 88:57–66.

Baik B-K, Ullrich SE (2008) Barley for food: Characteristics, improvement, and renewed interest. J. Cereal Sci. 48: 233-242.

Blandino M, Locatelli M, Sovrani V, Coïsson JD, Rolle L, Travaglia F, Giacosa S, Bordiga M, Scarpino V, Reyneri A, Arlorio M (2015) Progressive Pearling of Barley Kernel: Chemical Characterization of Pearling Fractions and Effect of Their Inclusion on the Nutritional and Technological Properties of Wheat Bread. J. Agric. Food Chem. 63:5875–5884.

Cavallero A, Gianinetti A, Finocchiaro F, Delogu G, Stanca AM (2004) Tocols in hull-less and hulled barley genotypes grown in contrasting environments. J. Cereal Sci. 39:175–180.

Corrêa TAF, Rogero MM, Hassimotto NMA, Lajolo FM (2019) The Two-Way Polyphenols-Microbiota Interactions and Their Effects on Obesity and Related Metabolic Diseases. Front. Nutr. 6:188.

De Graaff P, Govers C, Wichers HJ, Debets R (2018) Consumption of β-glucans to spice up T cell treatment of tumors: A review. Expert Opin. Biol. Ther. 18:1023-1040.

EFSA (2011). Scientific opinion on the substantiation of health claims related to β-glucans from oats and barley and maintenance of normal blood LDL-cholesterol concentrations (ID 1236, 1299), increase in satiety leading to a reduction in energy intake (ID 851, 852), reduction of post-prandial glycaemic responses (ID 821, 824), and "digestive function" (ID 850) pursuant to Article 13(1) of Regulation (EC) No 1924/ 2006. EFSA Journal, 9(6), 2207. Available online: https://www.efsa.europa.eu/en/efsajournal/pub/2207 [30 October 2020].

Fadel A, Mahmoud AM, Ashworth JJ, Li W, Ng YL, Plunkett A (2018) Health-related effects and improving extractability of cereal arabinoxylans. Int. J. Biol. Macromol. 109: 819–831.

Faostat. Available online: http://faostat.fao.org [20 January 2021].

FDA (2006). Draft Guidance for Industry and FDA Staff: Whole Grain Label Statements (Docket No. FDA-2006-D-0298). Issued by: Center for Food Safety and Applied Nutrition. Available online: https://www.regulations.gov/docket?D=FDA-2006-D-0298 [21 January 2021].

FDA (2008). Food Labeling: Health Claims, Soluble Dietary Fiber from Certain Foods and Coronary Heart Disease. Available online: https://www.federalregister.gov/documents/2008/02/25/E8-3418/food-labeling-health-claims-soluble-fiber-from-certain-foods-and-risk-of-coronary-heart-disease [30 October 2020].

Gamel T, Abdel-Aal E-SM (2012) Phenolic acids and antioxidant properties of barley wholegrain and pearling fractions. Agric. Food Sci. 21:118–131.

Gangopadhyay N, Harrison SM, Brunton NP, Hidalgo-Ruiz JL, Gallagher E, Rai DK (2018) Brans of the roller-milled barley fractions rich in polyphenols and health-promoting lipophilic molecules. J. Cereal Sci. 83:213–221.

Gangopadhyay N, Rai DK, Brunton NP, Gallagher E, Hossain, MB (2016) Antioxidant-guided isolation and mass spectrometric identification of the major polyphenols in barley (*Hordeum vulgare*) grain. Food Chem. 210:212–220.

Giordano D, Reyneri A, Locatelli M, Coïsson JD, Blandino M (2019) Distribution of bioactive compounds in pearled fractions of tritordeum. Food Chem. 301:125228.

Graebner RC, Wise M, Cuesta-Marcos A, Geniza M, Blake T, Blake VC, Butler J, Chao S, Hole DJ, Horsley R, Jaiswal P, Obert D, Smith KP, Ullrich S, Hayes PM (2015) Quantitative Trait Loci Associated with the Tocochromanol (Vitamin E) Pathway in Barley. PLoS ONE 10:e0133767.

Hassan AS, Houston K, Lahnstein J, Shirley N, Schwerdt JG, Gidley MJ, Waugh R, Little A, Burton RA (2017) A Genome Wide Association Study of arabinoxylan content in 2-row spring barley grain. PLoS ONE 12:e0182537.

Holtekjølen AK, Sahlstrøm S, Knutsen SH (2011) Phenolic contents and antioxidant activities in covered whole-grain flours of Norwegian barley varieties and in fractions obtained after pearling. Acta Agric. Scand. Sect. B Plant Soil Sci. 61:67–74.

Hong F, Yan J, Baran JT, Allendorf DJ, Hansen RD, Ostroff GR, Xing PX, Cheung N-KV, Ross GD (2004) Mechanism by Which Orally Administered β-1,3-Glucans Enhance the Tumoricidal Activity of Antitumor Monoclonal Antibodies in Murine Tumor Models. J. Immunol. 173: 797–806.

Huang D, Ou B, Hampsch-Woodill M, Flanagan JA, Prior RL (2002) High-Throughput Assay of Oxygen Radical Absorbance Capacity (ORAC) Using a Multichannel Liquid Handling System Coupled with a Microplate Fluorescence Reader in 96-Well Format. J. Agric. Food Chem. 50:4437–4444.

Idehen E, Tang Y, Sang S (2017) Bioactive phytochemicals in barley. J. Food Drug Anal. 25:148–161.

Irakli M, Lazaridou A, Mylonas I, Biliaderis CG (2020) Bioactive Components and Antioxidant Activity Distribution in Pearling Fractions of Different Greek Barley Cultivars. Foods 9:783.

Izydorczyk M, Dexter J (2008) Barley β-glucans and arabinoxylans: Molecular structure, physicochemical properties, and uses in food products—A Review. Food Res. Int. 41: 850–868.

Izydorczyk MS, Biliaderis CG (2007) Arabinoxylans: Technologically and Nutritionally Functional Plant Polysaccharides. In: CG Biliaderis, SM Izydorczyk (Ed.), Functional Food Carbohydrates; Taylor & Francis Group: Boca Raton, FL, USA, 2007; pp. 250–290.

Izydorczyk MS, McMillan T, Bazin S Kletke J, Dushnicky L, Dexter J, Chepurna A, Rossnagel B (2014) Milling of Canadian oats and barley for functional food ingredients: Oat bran and barley fibre-rich fractions. Can. J. Plant Sci. 94:573–586.

Lampi A-M, Nurmi T, Ollilainen V, Piironen V (2008) Tocopherols and Tocotrienols in Wheat Genotypes in the Healthgrain Diversity Screen. J. Agric. Food Chem. 56:9716–9721.

Lee C, Han D, Kim B, Baek N, Baik B-K (2013) Antioxidant and anti-hypertensive activity of anthocyanin-rich extracts from hulless pigmented barley cultivars. Int. J. Food Sci. Technol. 48:984–991.

Madhujith T, Shahidi F (2009) Antioxidant potential of barley as affected by alkaline hydrolysis and release of insoluble-bound phenolics. Food Chem. 117:615–620.

Malunga LN, Beta T (2015) Antioxidant Capacity of Water-Extractable Arabinoxylan from Commercial Barley, Wheat, and Wheat Fractions. Cereal Chem. J. 92:29–36.

Marconi E, Graziano M, Cubadda R (2000) Composition and Utilization of Barley Pearling By-Products for Making Functional Pastas Rich in Dietary Fiber and β-Glucans. Cereal Chem. J. 77:133–139.

Martínez M, Motilva MJ, López de las Hazas MC, Romero MP, Vaculova K, Ludwig IA (2018) Phytochemical composition and β-glucan content of barley genotypes from two different geographic origins for human health food production. Food Chem. 245:61–70.

Martínez-Subirà M, Romero MP, Puig E, Macià A, Romagosa I, Moralejo M (2020) Purple, high β-glucan, hulless barley as valuable ingredient for functional food. LWT 131: 109582.

Moreau RA, Flores RA, Hicks KB (2007) Composition of Functional Lipids in Hulled and Hulless Barley in Fractions Obtained by Scarification and in Barley Oil. Cereal Chem. J. 84:1–5.

Panfili G, Fratianni A, Di Criscio T, Marconi E (2008) Tocol and β-glucan levels in barley varieties and in pearling by-products. Food Chem. 107: 84–91.

Serra A, Rubio L, Macià A, Valls R-M, Catalán Ú, De La Torre R, Motilva M-J (2013) Application of dried spot cards as a rapid sample treatment method for determining hydroxytyrosol metabolites in human urine samples. Comparison with microelution solid-phase extraction. Anal. Bioanal. Chem. 405, 9179–9192.

Sharma P, Gujral HS (2014) Cookie making behavior of wheat-barley flour blends and effects on antioxidant properties. LWT 55:301–307.

Suriano S, Iannucci A, Codianni P, Fares C, Russo M, Pecchioni N, Marciello U, Savino M (2018) Phenolic acids profile, nutritional and phytochemical compounds, antioxidant properties in colored barley grown in southern Italy. Food Res. Int. 113:221–233.

Swanston JS, Ellis RP, Pérez-Vendrell A, Voltas J, Molina-Cano J-L (1997) Patterns of Barley Grain Development in Spain and Scotland and Their Implications for Malting Quality. Cereal Chem. J. 74: 456–461.

Thiex N, Novotny L, Crawford A (2012) Determination of Ash in Animal Feed: AOAC Official Method 942.05 Revisited. J. AOAC Int. 95:1392–1397.

Xia X, Xing Y, Kan J (2019) Antioxidant activity of Qingke (highland hull-less barley) after extraction/hydrolysis and in vitro simulated digestion. J. Food Process. Preserv. 44:14331.

Yoshida A, Sonoda K, Nogata Y, Nagamine T, Sato M, Oki T, Hashimoto S, Ohta H (2010) Determination of Free and Bound Phenolic Acids, and Evaluation of Antioxidant Activities and Total Polyphenolic Contents in Selected Pearled Barley. Food Sci. Technol. Res. 16:215–224.

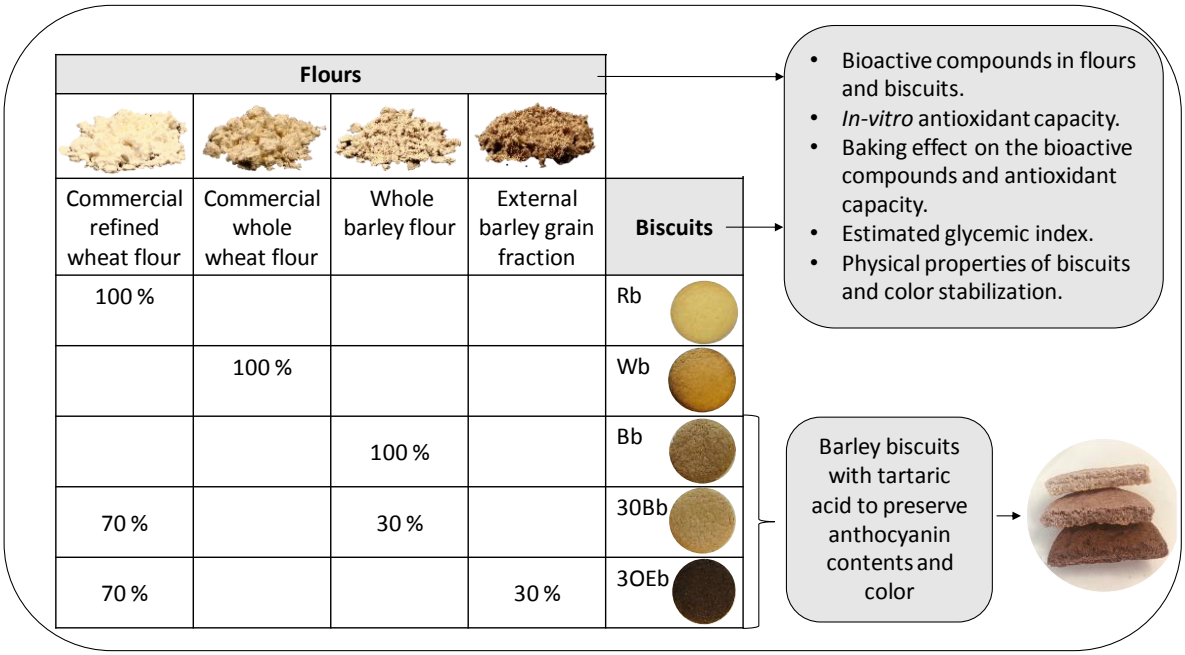
Zhang X-W, Jiang Q-T, Wei Y-M, Liu C (2017) Inheritance analysis and mapping of quantitative trait loci (QTL) controlling individual anthocyanin compounds in purple barley (*Hordeum vulgare* L.) grains. PLoS ONE 12:e0183704.

Zhu Y, Li T, Fu X, Abbasi AM, Zheng B, Liu RH (2015) Phenolics content, antioxidant and antiproliferative activities of dehulled highland barley (*Hordeum vulgare* L.). J. Funct. Foods 19:439–450.

Chapter V

Purple, high β-glucan, hulless barley as valuable ingredient for functional food*

M. Martínez-Subirà, M.-P. Romero, E. Puig, A. Macià, I. Romagosa, M. Moralejo



Graphical abstract

* Martínez-Subirà, M.; Romero, M.P.; Puig, E.; Macià, A.; Romagosa, I.; Moralejo, M.; Purple, high β-glucan, hulless barley as valuable ingredient for functional food. LWT - Food Sci. Technol. 2020, 131, 109582. https://doi.org/10.1016/j.lwt.2020.109582

LWT - Food Science and Technology 131 (2020) 109582

Contents lists available at ScienceDirect

LWT - Food Science and Technology

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

Purple, high β-glucan, hulless barley as valuable ingredient for functional food

Mariona Martínez-Subirà, M. Paz Romero, Eva Puig, Alba Macià, Ignacio Romagosa, Marian Moralejo*

University of Lleida - AGROTECNIO Center, Av. Rovira Roure 191, 25198, Lleida, Spain

ARTICLEINFO

Keywords:
Colored barley
Dietary fiber
Antioxidants
Healthy biscuits

ABSTRACT

Barley (*Hordeum vulgare* L.) stands out for its high content on bioactive compounds although it is not frequently found in human food. In this study, a purple hulless barley genotype was used to explore its food potential. β-glucans, arabinoxylans, anthocyanins and other phenolic components were determined in biscuits containing different proportions of whole barley flour and pearling fractions and compared to biscuits prepared with 100% refined (control) and 100% whole wheat flour. Barley biscuits were richer in bioactive compounds, showed higher *in-vitro* antioxidant capacity and lower estimated glycemic index with slight changes in physical properties. Baking did not affect β-glucans and arabinoxylans while it increased most of the phenolic compounds and antioxidant capacity. Barley anthocyanins were thermally unstable and exhibited high degradation rates but were partially stabilized by tartaric acid. Biscuits baked with 100% flour from purple barley grains fulfill the health claim of "high in fiber". A single biscuit provides more than 0.75g of β-glucans. Thus, one serving of four biscuits satisfies the 3g of β-glucans per day target to display the label of "reduces blood cholesterol and risk of heart disease".

V.1. Introduction

Barley is a cereal highly variable in its morphology, development, composition and adaptation. There are two- and six-rowed barleys according to the spike structure; hulled or hulless based on whether the hulls remain adhered to the grain or not; spring or winter cultivars with different requirements for flowering; diverse grain color (black, blue, purple or yellow) due to anthocyanins or other phenolic components. These barley types differ in their physical and chemical attributes and can be processed differently for diverse commercial purposes (Baik and Ullrich, 2008). Barley

* Corresponding author. Dpt. Chemistry University of Lleida - AGROTECNIO Center, Av. Rovira Roure 191, 25198, Lleida, Spain.
E-mail address: marian.moralejo@udl.cat(M. Moralejo).

https://doi.org/10.1016/j.lwt.2020.109582
Recceived 10 March 2020; Received in revised form April 2020; Accepted 9 May 2020
Available online 11 June 2020

grain is rich in starch (70-80%), has an adequate protein balance (10-16%) and low lipid content (2-3%). Most barley produced in the world is used for animal feed, less than 6% for malt and a relatively negligible quantity for food. However, barley is of increasing interest as a food due to its bioactive compounds. These include dietary fiber and phytochemical components such as phenolic acids, flavonoids, tocols, lignans, phytosterols and folates (Idehen, et al., 2017). Between them, β-glucans, arabinoxylans and some of phenolic compounds stand out by its positive roles in human health. β-glucans and arabinoxylans are non-starch polysaccharides and major constituents of cell walls. In barley, the β-glucan content varies between 3 and 11% and that of arabinoxylans from 2 to 9% (Hassan et al., 2017; Izydorczyk and Dexter, 2008). Both are dietary fiber and their intake has been related to health benefits. The American Food and Drug Administration released a health claim associating consumption of whole grain and dry milled barley products that provide at least 3g β-glucan per day with reduced total and LDL blood cholesterol (FDA, 2008). Furthermore, the European Food Safety Authority acknowledged than barley β-glucans reduce blood cholesterol and the risk of heart disease as well as contributing to a reduction in the rise of blood glucose after meal (EFSA, 2011). In recent years, β-glucans have also received increasing attention as immune system enhancers for fighting infectious diseases, inflammatory pathologies, and some types of cancer (Bashir and Choi, 2017; De Graaff et al, 2018; Hong et al., 2004). Arabinoxylans are also associated with lowering cholesterol and glucose as well as having antioxidant properties due to the presence of phenolic acids linked to their structure (Fadel et al., 2018; Izydorczyk and Dexter, 2008; Malunga and Beta, 2015). Barley is also a good source of phenolic compounds, which can be found free or bound to fiber. Phenolic compounds are known to reduce oxidative stress associated with metabolic diseases and to promote health by modulating degenerative pathologies such as cardiovascular disease, inflammation and cancer (Calinoiu and Vodnar, 2018). Flavonoids are the main compounds in the free phenolic fraction, while phenolic acids are the most abundant among the bound one. Anthocyanins, which belong to the class of flavonoids, are normal constituents in colored barley grains. Due to its health benefits, particularly from β-glucans, barley has been tested as an ingredient to improve the nutritional value of such wheat-based products as cookies (Sharma and Gujral, 2014) or bread (Blandino et al., 2015). However, not much attention has been paid to specific types of barleys that may have particular interest for the production of healthy foods. This is the case of the colored genotypes rich in anthocyanins, which are strongly correlated with antioxidant properties (Kahkonen and Heinonen, 2003). As far as we know, the use of purple barley in the production of functional foods has not been reported. Therefore, in this study, a purple, hulless genotype was selected to explore its potential for food purposes through the following tasks: 1) determining the β-glucan, arabinoxylan and phenolic compound composition; 2) preparing biscuits containing barley flour or external barley grain fractions, analyzing their composition, and evaluating the impact of baking on the bioactive compounds compared with refined and whole wheat flour biscuits; 3) assessing their *in-vitro* antioxidant capacity, estimated Glycemic Index (eGI), and physical properties.

V.2. Materials and methods

V.2.1. Plant material

Hindukusch, an Afghan barley landrace, purple in color and with hulless grains, was used for this study (https://www.seedstor.ac.uk/search-infoaccession.php?idPlant=3707).

V.2.2. Flour and barley fractions

Whole grain was ground in a Foss Cyclotec 1093™ mill equipped with a 0.5-mm screen (FOSS, Barcelona, Spain). The external grain fraction was obtained by pearling until a loss of 15% of the original grain weight, using a TM-05C pearling machine (Satake, Thailand CO., LTD) at 1060 rpm.

V.2.3. Biscuits elaboration

Biscuits were prepared according to the AACC method 10-50.05 (AACC, 2008) with little modifications, using 112.5g flour, 32g vegetable margarine, 65g sugar, 1g salt, 1.25g sodium bicarbonate, 16.5g 6% dextrose solution and 8g distilled water. On a dry weight basis, flour represented 53% of the biscuit formulations. Flours and blends to prepare the biscuits were: R (100% commercial refined wheat flour), W (100% commercial whole wheat flour), B (100% whole barley flour), 30B (70% R: 30% B), 30E (70% R: 30% external barley grain fraction). The ingredients were mixed in a spiral kneader for 6 min. The dough was flattened to 6 mm, cut into 60 mm diameter pieces, and baked in an industrial oven (PE 46 SVR, Eurofred, Italy) at 200 °C for 10 min. The biscuits were cooled for 30 min, stored in an airtight plastic container and kept at room temperature prior to chemical and physical analysis. They were coded according to their flour name followed by letter b.

V.2.4. β-glucan, arabinoxylan and amylose determination

β-glucan, arabinoxylan and amylose content of starches were determined using mixed-linkage β-glucan assay (K-BGLU), D-xylose assay (K-XYLOSE) and amylose/amylopectin assay (K-AMYL) kits from Megazyme (Wicklow, Ireland).

V.2.5. Phenolic compounds (PC) and anthocyanin analysis by UPLC-MS/MS

Free and bound PCs were extracted according to Martínez et al. (2018). Extracts were analyzed in an AcQuity Ultra-Performance TM liquid chromatography coupled to a tandem mass spectrometer (UPLC-MS/MS) from Waters (Milford, MA, USA). The analytical column was an AcQuity BEH C18 column (100 mm × 2.1 mm i. d., 1.7 µm) equipped with a Van Guard TM Pre-Column AcQuity BEH C18 (5 mm × 2.1 mm, 1.7 µm), also from Waters. The mobile phase was 0.2% (v/v) acetic acid and acetonitrile for phenolic compounds (PC), and 10% acetic acid (v/v) and acetonitrile for anthocyanins. The HPLC system was coupled to a triple quadrupole detector mass spectrometer (Waters, Milford, MA, USA) equipped with a Z-spray electrospray interface for ionization, operating in negative mode $[M-H]^-$ for PCs, and positive mode $[M-H]^+$ for anthocyanins. Phenolic compounds were quantified by reference to a 0.02-25 ng calibration curve of commercially available standard compounds and results expressed as µg/g dry sample. A linear response was obtained for all the available standards, as checked by linear regression analysis. Limits of detection (LOD) ranged from 0.007 to 0.09 ng and limits of quantification (LOQ), from 0.02 to 0.30 ng.

V.2.6. *In-vitro* antioxidant capacity

The Oxygen Radical Absorbance Capacity (ORAC) of the PC extracts was measured according to Huang et al, (2002). Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) was used as the control, with one ORAC unit being equal to the antioxidant protection given by 1 µmol Trolox. The antioxidant capacity was determined using a FLUOstar OPTIMA fluorescence reader (BMG Labtech), with reagents automatically transferred into a 96-well flat-bottom polystyrene microplate controlled by the OPTIMA 2.10R2 software. The reader was equipped with fluorescence filters with 485 nm excitation and 520 nm emission wavelengths in order to measure changes in the

fluorescence of fluorescein under controlled temperature (37 °C). The total antioxidant capacity was expressed as µmols of Trolox-equivalents per g of dry sample.

V.2.7. Estimated glycemic index

In-vitro digestibility was evaluated according to Brennan and Tudorica (2008). A non-linear model was applied to describe the kinetics of starch hydrolysis (Goñi, et al., 1997). The area under hydrolysis curve (AUC) was calculated using the equation: $[AUC = C_{\infty}(t_f - t_0) - \left(\frac{C\infty}{k}\right)[1 - e^{-k(tf - t0)}]$; where C∞ corresponds to the concentration at equilibrium (t_{180}), t_f to final time (180 min), t_0 to initial time (0 min), and k to kinetic constant. The hydrolysis index (HI) was calculated from the AUC of the samples as percentage of the corresponding area of the reference white wheat bread [HI = AUC sample/AUC wheat bread*100]. The estimated glycemic index (eGI) was finally determined using the equation [eGI = 0.862*HI + 8.198] (Granfeldt, 1994).

V.2.8. Physical parameters

Width (W) and thickness (T) were measured according to the 10-50.05 AACC method. The texture analyzer TX-XT2 (Stable Micro Systems, Ltd. USA) with a cylindrical puncture probe (P/2) was used to measure strength. Hardness (N) was measured from the force-distance curves and calculated by the SMS Exponent Connect software. The L*, a* and b* chromatic values were measured with a Macbeth Color-eye 3000 colorimeter (Altrincham, UK). Spectral data were obtained using an Illuminator C and a 10° observer.

V.2.9. Statistical analysis

All measurements were carried out in triplicate. The analysis was conducted with JMP®Pro14 (SAS institute Inc., Cary NC). Tukey-Kramer's HSD (α = 0.05) was preferred for multiple comparisons.

V.3. Results and discussion

V.3.1. Bioactive compounds in flours and biscuits

The bioactive composition of the flours and biscuits prove the interest of purple hulless barley genotypes as a valuable ingredient for healthy food products. Table V.1 shows the β-glucan and arabinoxylan contents of the flours and biscuits. Barley flour (B) was high in β-glucans. The β-glucan content of refined wheat flour (R) increased over ten folds when mixed with 30% whole barley flour (30B). The incorporation of 30% barley external fraction did not increase the β-glucans in refined wheat flour due to the low β-glucan content of the outer external layers of the grain. After biscuit making, the 100% barley biscuit (Bb) was the richest in β-glucans. The refined wheat biscuit (Rb) increased tenfold its content in β-glucans with the inclusion of 30% whole barley flour (30Bb). No significant differences were found between the β-glucan content of Rb, whole wheat biscuits (Wb), and biscuits containing barley external fractions (30 Eb). As each biscuit weighed about 20g, Bb provides more than 0.75g of β-glucans per unit, which is the limit defined by EFSA for labelling (EFSA, 2011). Additionally, one serving of four Bb biscuits would satisfy the 3g of β-glucans per day goal to support the claim of reducing blood cholesterol and the risk of heart disease (EFSA, 2011). Concerning arabinoxylans, its content in the barley flour was similar to whole wheat (W) and twice that of the control. The arabinoxylan content detected in Hindukusch was in agreement with those previously reported in the literature for barley grain, being determined by genetic factors but also affected by the environmental conditions (Hassan et al., 2017; Izydorczyk and Dexter, 2008). In contrast to β-glucans, arabinoxylans are predominant in the outer layers of the grain and 30 Eb biscuit was the richest in arabinoxylans, with similar values than those from Wb and four times

higher than the control. Regarding β-glucans and arabinoxylans, the major components of the barley dietary fiber, Bb also fulfilled the claim of "high in fiber" for products containing at least 6g fiber/100g food. Wb, 30Bb and 30 Eb could also be considered as "source of fiber", since they contained more than 3g fiber/100g food (Regulation EC No 1924/2006).

Table V.1. β-glucans and arabinoxylans contents detected in flours and biscuits.

	β-glucans		Arabinoxylans	
Flours	**g /100 g flour**			
R	0.22	c	3.14	c
W	0.83	c	7.01	ab
B	8.26	a	6.26	b
30B	2.61	b	3.83	c
30E	1.10	c	8.27	a
SED	*0.31*		*0.44*	
Biscuits	**g /100 g biscuit**			
Rb	0.14	c	1.51	d
Wb	0.40	c	4.15	ab
Bb	4.26	a	3.26	bc
30Bb	1.12	b	2.27	cd
30Eb	0.58	c	4.40	a
SED	*0.12*		*0.29*	

Results as mean of three replicates. Values followed by different letters are significantly different acording to Tukey-Kramer's HSD (0.05); SED, standard error of the difference.

Hindukusch was richer in phenolic compounds (PC) than both wheat flours (Table V.2). The phenolic content of refined flour was very low, but when 30% of barley external fraction was added, the concentration increased to similar value to the 100% barley flour (30E). This was not observed when 30% of B was added to W (30B) because PC are mainly located in the outer layers of the barley grain like in others cereals (Fardet, et al., 2008). Analysis of phenolic profile of flours and biscuits was carried out by UPLC-MS/MS. Thirty-six different compounds were identified, flavanols and phenolic acids being the most abundant. Negligible amounts of free PCs were observed in the control flour. Hindukusch contained 304 μg/g sample of total free PCs among which proanthocyanidins and catechins accounted for 65%, phenolic acids for 26% and flavone glycosides for 9%. Bound phenolic acids were predominant in all flours, representing 74-99% of total PC. Between them, ferulic acid was the main component with 76-87% of the total. Hindukusch contained twelve and three times greater that of the R and W flours respectively. The biscuits with the highest content of PC were Bb and 30 Eb, followed in decreasing order by 30Bb, which had similar content to Wb. Free PCs ranged from 30 μg/g sample in Rb to 163 μg/g sample in Bb. Bb and 30 Eb contained the highest amount of bound PCs, while Rb had the lowest. Anthocyanins were minor constituents compared to the phenolic acids and flavanols (Table V.3). A total of 22 anthocyanins were detected in Hindukusch. These comprised pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvinidin conjugates of glucose, acetylglucose, malonylglucose, dimalonyglucose, dihexose and rutinose (Table V.S.1). The Hindukusch anthocyanins content agreed with earlier studies on purple barley cultivars (Martínez et al., 2018; Zhang et al., 2017), but was lower than that reported by Lee et al. (2013), indicating a broad diversity of concentrations associated with environmental and genetic factors. The most abundant anthocyanins were cyanidin dimalonylglucoside (45%) and cyanidin glucoside (35%). As anthocyanins are located in the pericarp and aleurone layers, flour containing the external

fraction had the highest amount of anthocyanins. Thus, 30 Eb biscuits showed the highest anthocyanin content, followed by Bb and 30Bb. We are not aware of previous data on the chemical composition of foods containing purple barley flour, but the anthocyanin content detected in 30 Eb was higher than that found in other functional biscuits prepared with purple wheat flour (Pasqualone et al.,2015).

Table V.2. Content of Free and Bound Phenolic Compounds (PC) and Antioxidant Capacity detected in flours and biscuits.

	Flours						Biscuits					
	R	**W**	**B**	**30B**	**30E**	***SED***	**Rb**	**Wb**	**Bb**	**30Bb**	**30Eb**	***SED***
Free PC			**µg /g flour**						**µg /g biscuit**			
Catechin	-	-	23.8 ^{a}	6.6 ^{c}	14.0 ^{b}	*1.18*	2.4 ^{b}	-	20.4 ^{a}	4.2 ^{b}	17.1 ^{a}	*2.42*
Catechin-glucoside	-	-	20.3 ^{a}	6.4 ^{b}	18.1 ^{a}	*1.35*	-	-	2.3 ^{a}	-	-	*0.35*
Procyanidin B3	-	-	71.6 ^{a}	20.6 ^{b}	85.0 ^{a}	*4.72*	3.7 ^{b}	2.4 ^{b}	8.8 ^{a}	-	3.6 ^{b}	*0.60*
GC-C/Prodelphinidin B4	-	-	72.0 ^{b}	23.4 ^{c}	116.6 ^{a}	*1.25*	2.6 ^{c}	3.1 ^{bc}	9.2 ^{a}	2.8 ^{c}	4.1 ^{b}	*0.31*
Other minorities	-	-	10.2 ^{b}	3.0 ^{c}	17.6 ^{a}	*0.96*	-	-	10.4 ^{a}	2.7 ^{b}	10.3 ^{a}	*0.77*
Total flavanols	-	-	197.9 ^{b}	60.0 ^{c}	251.2 ^{a}	*7.03*	8.7 ^{c}	5.5 ^{c}	51.1 ^{a}	9.7 ^{c}	35.1 ^{b}	*3.38*
p-OHBenzoic acid	nq	nq	12.6 ^{a}	11.0 ^{b}	13.2 ^{a}	*0.18*	9.8 ^{b}	9.8 ^{b}	12.0 ^{a}	10.4 ^{b}	13.4 ^{a}	*0.44*
2,4-DiOHBenzoic acid	-	nq	14.6 ^{a}	nq	16.19 ^{a}	*0.51*	-	12.2 ^{b}	19.8 ^{ab}	13.9 ^{b}	26.3 ^{a}	*2.49*
Coumaric acids	-	-	1.9 ^{b}	-	2.7 ^{a}	*0.13*	nq	1.5 ^{c}	3.1 ^{b}	3.7 ^{b}	5.8 ^{a}	*0.29*
Vanillic acid	nq	3.4 ^{c}	15.1 ^{b}	5.4 ^{c}	18.0 ^{a}	*0.77*	-	3.2 ^{b}	13.0 ^{a}	4.8 ^{b}	17.5 ^{a}	*1.46*
Ferulic acids	nq	3.7 ^{c}	3.7 ^{c}	5.2 ^{b}	6.6 ^{a}	*0.41*	10.7 ^{d}	20.3 ^{cd}	39.5 ^{b}	30.1 ^{bc}	70.0 ^{a}	*5.15*
Gallic acid	-	15.1 ^{c}	21.7 ^{a}	15.9 ^{c}	18.7 ^{b}	*0.49*	-	-	-	-	-	
Other minorities	-	7.6 ^{b}	8.2 ^{a}	6.2 ^{c}	8.1 ^{a}	*0.16*	-	8.5 ^{a}	0.8 ^{b}	nq	8.7 ^{a}	*0.49*
Total phenolic acids	-	29.7 ^{d}	77.7 ^{b}	43.8 ^{c}	83.4 ^{a}	*1.23*	20.5 ^{d}	55.4 ^{c}	88.3 ^{b}	62.9 ^{bc}	141.6 ^{a}	*9.65*
Luteolin-O-glucoside	-	-	8.6 ^{b}	7.3 ^{c}	9.6 ^{a}	*0.26*	-	-	7.4 ^{b}	6.6 ^{c}	8.3 ^{a}	*0.22*
Isoscoparins	-	-	16.9 ^{a}	14.1 ^{c}	15.3 ^{b}	*0.17*	-	-	14.2 a	6.5 ^{b}	13.5 ^{a}	*0.30*
Other minorities	0.8 ^{c}	10.4 ^{a}	2.6 ^{bc}	1.9 ^{bc}	5.1 ^{b}	*1.06*	1.1 ^{c}	4.7 ^{a}	1.6 ^{c}	0.8 ^{c}	2.6 ^{b}	*0.25*
Total flavone glycosides	0.8 ^{d}	10.4 ^{a}	28.1 ^{a}	23.3 ^{b}	30.1 ^{a}	*1.06*	1.1 ^{d}	4.7 ^{c}	23.2 ^{a}	13.9 ^{b}	24.4 ^{a}	*0.68*
Total free phenols	**0.8** ^{e}	**40.1** ^{d}	**303.8** ^{b}	**127.0** ^{c}	**364.7** ^{a}	*8.02*	**30.2** ^{c}	**65.6** ^{bc}	**162.6** ^{a}	**86.4** ^{b}	**201.1** ^{a}	*13.40*
Bound PC			**µg /g flour**						**µg /g biscuit**			
p-OHBenzoic acid	3.0 ^{c}	4.4 ^{c}	10.5 ^{b}	5.5 ^{c}	14.8 ^{a}	*0.92*	3.3 ^{b}	6.8 ^{b}	13.1 ^{a}	4.8 ^{b}	12.8 ^{a}	*1.24*
OHBenzoic acid	4.6 ^{a}	4.4 ^{a}	6.3 ^{a}	6.5 ^{a}	4.8 ^{a}	*1.42*	5.1 ^{a}	5.7 ^{a}	4.8 ^{a}	5.4 ^{a}	5.7 ^{a}	*0.52*
2,4-DiOHBenzoic acid	-	-	5.0 ^{b}	-	17.9 ^{a}	*0.33*	-	3.4 ^{b}	8.9 ^{a}	2.2 ^{b}	11.2 ^{a}	*1.17*
Coumaric acids	3.0 ^{c}	13.6 ^{bc}	130.2 ^{a}	32.9 ^{b}	149.3 ^{a}	*8.77*	16.1 ^{c}	55.3 ^{c}	136.3 ^{b}	22.6 ^{c}	218.8 ^{a}	*20.45*
Vanillic acid	1.2 ^{c}	6.2 ^{bc}	31.6 ^{a}	9.8 ^{b}	37.0 ^{a}	*2.22*	4.6 ^{b}	17.4 ^{b}	38.7 ^{a}	8.5 ^{b}	43.1 ^{a}	*4.48*
Ferulic acids	73.1 ^{c}	288.3 ^{b}	822.9 ^{a}	318.7 ^{b}	795.1 ^{a}	*50.38*	290.5 ^{b}	485.2 ^{b}	919.5 ^{a}	416.8 ^{b}	941.3 ^{a}	*73.05*
Other minorities	2.6 ^{e}	16.1 ^{c}	21.9 ^{b}	9.4 ^{d}	28.1 ^{a}	*0.52*	8.7 ^{b}	14.3 ^{b}	27.7 ^{a}	12.9 ^{b}	24.2 ^{a}	*1.94*
Total phenolic acids	87.7 ^{c}	332.9 ^{b}	1028.4 ^{a}	382.8 ^{b}	1047.0 ^{a}	*61.79*	328.3 ^{b}	588.0 ^{b}	1148.8 ^{a}	473.2 ^{b}	1257.1 ^{a}	*98.95*
Total flavone glycosides	-	-	1.8 ^{a}	-	1.7 ^{a}	*0.02*	1.6 ^{b}	0.04 ^{c}	1.7 ^{ab}	-	1.8 ^{a}	*0.06*
Total bound phenols	**87.7** ^{c}	**332.9** ^{b}	**1030.2** ^{a}	**382.8** ^{b}	**1048.7** ^{a}	*61.79*	**329.9** ^{b}	**588.0** ^{b}	**1150.5** ^{a}	**473.2** ^{b}	**1258.8** ^{a}	*98.99*
Total phenolic compounds	**88.5** ^{c}	**373.1** ^{b}	**1334.0** ^{a}	**509.8** ^{b}	**1413.4** ^{a}	*59.78*	**360.1** ^{b}	**653.6** ^{b}	**1313.1** ^{a}	**559.6** ^{b}	**1459.9** ^{a}	*95.89*
Antioxidant Capacity			**µmol Trolox /g flour**						**µmol Trolox /g biscuit**			
Total Antioxidant Capacity	**22.0** ^{c}	**44.5** ^{b}	**112.3** ^{a}	**47.9** ^{b}	**113.5** ^{a}	*2.23*	**18.1** ^{d}	**34.6** ^{c}	**68.1** ^{b}	**33.7** ^{c}	**92.6** ^{a}	*3.31*

Results as mean of three replicates. For each type of product, flours and biscuits, values within a row followed by different letters indicate significant differences acording to Tukey-Kramer's HSD (0.05); SED, standard error of the difference; - not detected (< LOD); nq, not quantified (< LOQ).

Table V.3. Anthocyanins content detected in flour and biscuits

	Pelargonidins	Cyanidins	Peonidins	Delphinidins	Petunidins	Malvidins	Total
Flours				**µg /g flour**			
B	4.48 b	30.17 b	0.040 b	2.12 b	0.142 b	0.27 a	37.21 b
30B	1.30 c	12.62 c	-	0.63 c	0.032 c	0.06 b	14.65 c
30E	7.03 a	65.43 a	0.064 a	3.27 a	0.202 a	0.22 a	76.22 a
SED	*0.223*	*1.659*	*0.003*	*0.209*	*0.014*	*0.019*	*1.673*
Biscuits				**µg /g biscuit**			
Bb	0.70 c	9.96 b	-	0.40 b	0.046 ab	0.02 bc	11.14 b
30Bb	0.26 d	4.04 c	-	0.08 c	0.001 b	-	4.38 c
30Eb	0.80 c	11.49 ab	0.044 b	0.38 b	0.035 ab	0.02 c	12.76 b
Bb + T*	1.67 a	13.39 a	0.002 b	0.88 a	0.050 ab	0.08 a	16.07 a
30Bb + T*	0.31 d	3.17 c	0.050 b	0.21 c	0.010 b	0.01 c	3.77 c
30Eb + T*	1.50 b	13.39 a	0.237 a	0.92 a	0.055 a	0.03 b	16.14 a
SED	*0.034*	*0.572*	*0.015*	*0.041*	*0.009*	*0.004*	*0.591*

Results as mean of three replicates. Values followed by different letters are significantly different acording to Tukey-Kramer's HSD (0.05). SED, Standard Error Difference; - not detected (< LOD); + T* biscuits with tartaric acid (0.5 g /100 g sample).

V.3.2. *In-vitro* antioxidant capacity

The ORAC values followed a similar tendency to that observed for the PC, indicating a positive correlation between the two assays (Table V.2). The antioxidant capacity of refined flour increased five times with the incorporation of 30% barley external fraction. The 30 Eb biscuits showed the highest antioxidant capacity exceeding that of the Bb despite having similar PC content. This could be attributed to its higher content in Maillard products such as melanoidins with known antioxidant capacity, which also corresponds to its higher observed brown color. The ORAC values observed in barley biscuits were similar to those found in some fruit and vegetables measured by the same methodology (Wu et al., 2004). Although the *in-vitro* antioxidant capacity may be different to the *in-vivo*, it can be estimated that cereal products would provide 60-70% of their potential antioxidant capacity based on the regular consumption to protect from oxidative stress disorders (Fardet et al., 2008). Thus, grain varieties with high antioxidant contents for food purposes can be useful in populations where fruit and vegetables are not often consumed.

V.3.3. Association between variables

Figure V.1 shows the Principal Component Analysis of the bioactive compounds and antioxidant capacity of biscuits. The two principal components together explained 94% of the variability in the standardized data set. PCA1, explaining 78% of the variation, seems related to the phenolics and antioxidant capacity whereas PCA2, explaining 16%, was linked to dietary fiber, β-glucans being negatively correlated with arabinoxylans. Bb had the highest β-glucan content while Wb and 30 Eb were the richest in arabinoxylans. Bb and 30 Eb showed the highest amount in anthocyanins and PCs as well as the highest antioxidant capacity, although their phenolic profiles differed; Bb had more flavanols than 30 Eb, while 30 Eb was richer in free phenolic acids (Table V.2). For PC and antioxidant capacity, the best choice was 30 Eb, but the best compromise was shown by the Bb biscuits as these were simultaneously high in β-glucans, PCs and antioxidant capacity.

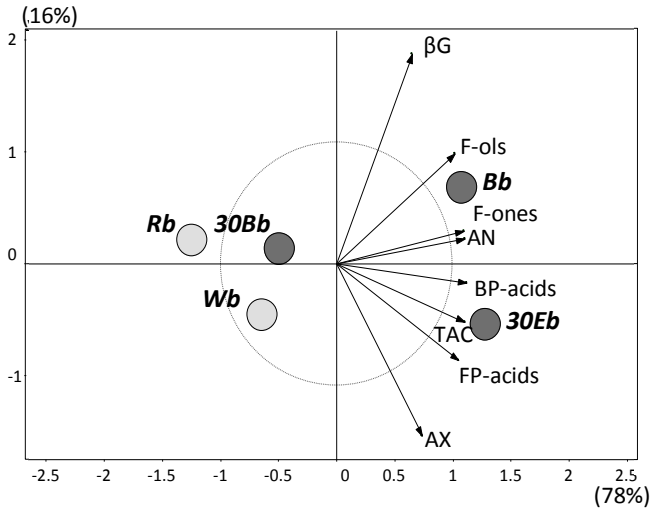


Figure V.1. Principal Component Analysis of biscuits for bioactive compounds and antioxidant capacity. AN, anthocyanins; AX, arabinoxylans; βG, β-glucans; BP-acids, bound phenolic acids; F-ols, flavonols; F-ones, flavone-glycosides; FP-acids, free phenolic acids; TAC, total antioxidant capacity.

V.3.4. Effect of the baking on the bioactive compounds and antioxidant capacity

In order to compare the bioactive concentrations in flour and biscuits directly, the absolute values recorded in biscuits should be divided by 0.53 as this is the proportion of flour, on a dry weight basis, they contained. Figures V.2A,B shows the absolute β-glucan and arabinoxylan contents of flours and the relative contents in biscuits. The values were close to those expected based on their percentage in flours, indicating that baking did not degrade β-glucans or arabinoxylans. These results were in accordance with those observed in other foods containing barley (Vashantan et al.,2002; Trogh et al., 2004; Mosele et al., 2018).

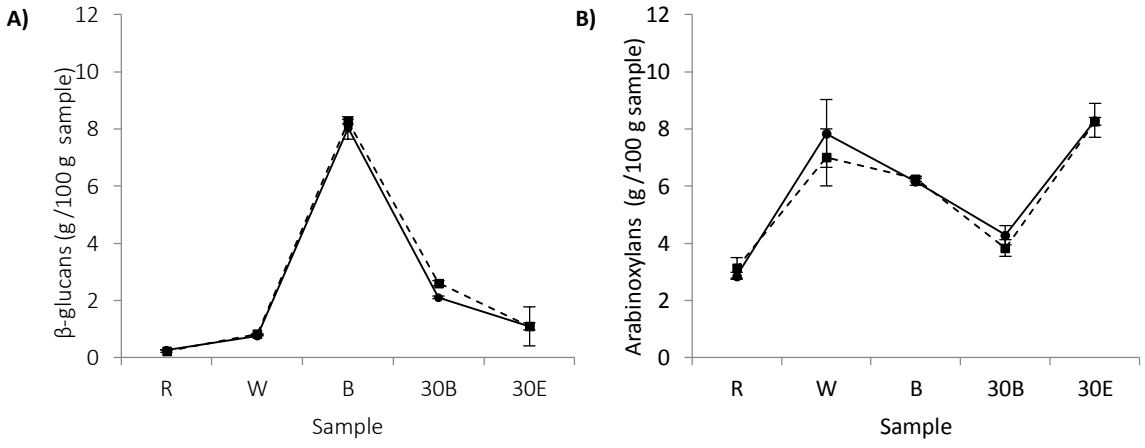


Figure V.2. (A) β-glucans content in flours (g/100 g sample) and biscuits (g/100 g flour in biscuits), **(B)** Arabinoxylans content in flour (g/100 g sample) and biscuits (g/100 flour in biscuits). Bars constructed using ± Std Error, n = 3. - - ■ - - flours, ——●—— biscuits*. (*) Relative values adjusted by the percentage of flour (dwb) contained in biscuits.

Biscuits contained a higher PC content than expected from the percentage of flour. However, baking affected individual compounds differently. Total free PCs remained stable due to the compensation between the increase in the phenolic acids, aldehydes and flavone glycosides and the decrease of most flavanols (Figure V.3A; Table V.2). Some authors have reported that thermal treatments may have a positive or negative influence on free PCs during processing. The final result is the balance between the decarboxylation of phenolic acids by exposure to high temperatures (Wani and Kumar, 2016) and their release from the fibre. Baking also increased bound PCs, especially coumaric and ferulic acids, in all biscuits (Figure V.3A; Table V.2). The most abundant phenolics in grains are bound to the fibre and can be released when exposed to thermal treatments. In addition, despite the sequential extraction of free and bound PCs from flours, some compounds may not be fully extracted and thus, the total components may be underestimated (Adom and Liu, 2002; Koddami et al., 2013). The antioxidant capacity increased significantly after baking, as did the PCs (Figure V.3B; Table V.2). This can be attributed to the above mentioned release of phenolic acids from the food matrix, to the formation of some products from the Maillard reaction, or to the presence of other products newly formed after thermal treatment exhibiting antioxidant activity sometimes superior to that of native molecules (Chaaban et al., 2016).

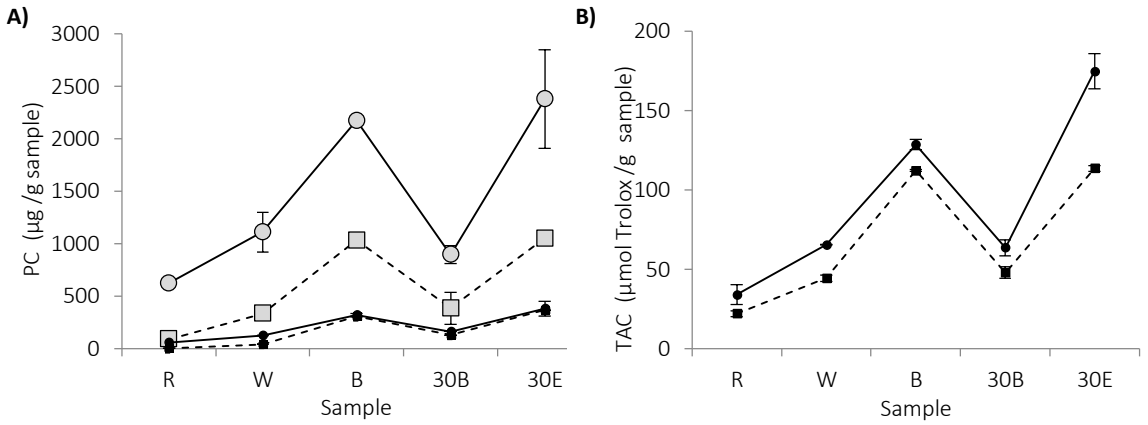


Figure V.3. (A) Phenolic compounds (PC) in flours (µg/g sample) and biscuits (µg/g flour in biscuit). Bars constructed using ± Std Error, n = 3. Bound PC flours, Bound PC biscuits*, Free PC flours, Free PC biscuits*. **(B)** Total antioxidant capacity (TAC) in flours (µmol Trolox/g sample) and biscuits (µmol Trolox/g flour in biscuit). Bars constructed using ± Std Error, n = 3. flours, biscuits*.(*) Relative values adjusted by the percentage of flour (dwb) contained in biscuits.

Anthocyanins are thermal unstable compounds and thus, decreased after baking. Barley biscuits showed lower anthocyanin contents than their corresponding flours (44% less in Bb, 44% in 30Bb and 68% in 30 Eb; Figure V.4). Malvidins were the most unstable anthocyanins (89% degradation), followed by delphinidins (73%), pelargonidins (71%), petunidins (50%) and cyanidins (48%). Similar results have been reported in colored corn biscuits, and these were attributed to baking above 180°C (Žilić et al., 2016). Thermal degradation begins with the hydrolysis of the sugar moieties, which are then degraded to chalcones. Subsequent breakdown of chalcones results in the formation of phenolic acids and carboxyaldehydes (Sui et al., 2015). It has been suggested that the degradation products retain antioxidant properties and hence, thermal degradation may not have a significant impact on the antioxidant capacity of the final product (Slavin et al., 2013).

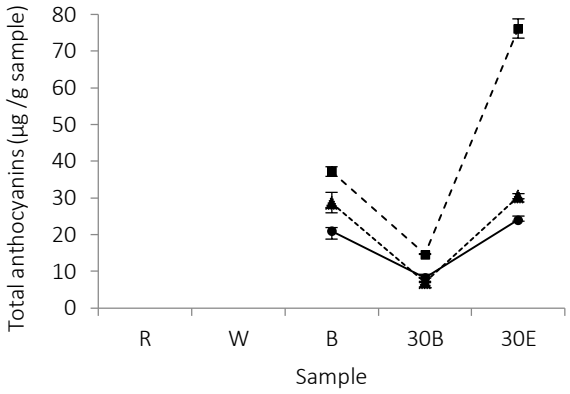


Figure V.4. Total anthocyanins content in flours (µg/g sample) and biscuits (µg/g flour in biscuit). Bars constructed using ± Std Error, n = 3. flours, biscuits*, biscuits with tartaric acid* (0.5 g/100 g sample). (*) Relative values adjusted by the percentage of flour (dwb) contained in biscuits.

V.3.5. Estimated glycemic index

Reducing the glycemic index is of interest in widely consumed cereal products, which are classified as moderate (55-70) to high (> 70) glycemic index foods (Atkinson et al., 2008). In this study, an *in-vitro* methodology was used to elucidate whether purple barley flour or its fractions decreased the eGI of wheat biscuits. Table V.4 shows the eGI of the biscuits and % amylose in starches. The control and Wb showed eGIs of 55.7 and 55.3 respectively. Substituting refined wheat with 30% barley flour or fractions reduced the eGI to 53.4 and 52.3 respectively, which ranked the products among the low glycemic index foods. The lowest eGI was 50.8 detected in the Bb biscuit. The raw materials and baking process influence the eGI values; dietary fiber and high amylose starch contribute to lower glycemic index (Fardet et al., 2006). The eGI of barley biscuits can be attributed to their higher β-glucan and arabinoxylan contents. It is known that both components increase matrix viscosity and hinder the ability of digestive enzymes to release glucose resulting in a reduced glycemic response (Brennan and Tudorica, 2008). However, the results cannot be exclusively explained by their fiber content but also by other factors, such as the amylose/amylopectin ratio since amylose is more resistant to alpha-amylases (Sajilata et al., 2006). This may explain why the Wb had a higher eGI than 30Bb and 30 Eb biscuits despite having a similar total fiber content.

Table V.4. Starch amylose (%) and estimated Glycemic Index (eGI) for biscuits

Amylose (%)		**eGI**	
Flours		**Bisuits**	
R	22.6 a	Rb	55.7 a
W	12.4 b	Wb	55.3 a
B	20.1 a	Bb	50.8 c
30B	22.1 a	30Bb	53.4 b
30E	15.3 b	30Eb	52.3 bc
SED	*0.94*		*0.49*

Results as mean of three replicates. Values followed by different letters indicate significant differences acording to Tukey-Kramer's HSD (0.05). SED, Standard Error Difference.

V.3.6. Physical properties of biscuits and color stabilization

The physical characteristics of biscuits are shown in Table V.5. The 30Bb had similar volume and density to the control and was softer than Wb. The Bb and 30 Eb barley biscuits had a lower volume and more density than the control but similar hardness to Wb. Regarding color, significant differences were found in the L*, a* and b* values. The control biscuits were the lightest (L* = 76), followed by the Wb > 30Bb > Bb > 30 Eb. This order was associated with their corresponding contents in anthocyanins and other PCs. All barley biscuits were less reddish (a*) and yellowish (b*) than wheat biscuits due to the high pH caused by the bicarbonate used as leavening agent. In order to stabilize their purple color, the pH was lowered from 8 to 6 by adding 0.5g/100g tartaric acid to the dough and the new neutral conditions allowed lighter and redder barley biscuits without other significant physical changes (Table V.5). In addition, not only was the color preserved but also a lower reduction in the anthocyanin content after baking was observed in Bb (19%) and 30 Eb (60%) (Fig. 4). Hence, the choice of leavening agent seems to be a critical factor for the stability of anthocyanins during the baking process. More research is needed to evaluate the effect of other acidulates and their concentrations on the anthocyanin content of barley biscuit formulations.

Table V.5. Physical parameters of wheat and barley biscuits.

	Volume cm³	Density g /cm³	Hardness N	Colour		
				L*	a*	b*
Rb	41.2 a	0.48 c	10.2 d	75.7 a	3.5 c	14.0 a
Wb	39.1 b	0.54 a	23.0 ab	70.2 b	4.1 b	11.3 b
Bb	34.8 cd	0.55 a	24.2 a	54.4 f	0.3 e	6.3 d
30Bb	41.7 a	0.45 c	18.3 c	61.4 d	0.7 e	9.2 c
30Eb	37.6 bc	0.50 bc	23.0 ab	48.5 g	0.4 e	5.8 d
Bb + T*	34.4 d	0.54 a	24.5 a	56.5 e	5.8 a	9.5 c
30Bb + T*	42.7 a	0.46 c	19.4 bc	64.8 c	2.6 d	11.9 b
30Eb + T*	37.7 bc	0.53 ab	22.9 ab	53.5 f	6.2 a	9.2 c
SED	*0.76*	*0.01*	*1.18*	*0.26*	*0.16*	*0.36*

Results as mean of three replicates. Values followed by different letters indicate significant differences acording to Tukey-Kramer's HSD (0.05). SED, Standard Error Difference; + T* biscuits with tartaric acid (0.5 g /100 g sample).

V.4. Conclusions

Biscuits containing purple barley ingredients were richer in bioactive compounds, showed higher antioxidant capacity and lower eGI than refined and whole-wheat biscuits. Their physical characteristics were slightly worse than the Rb but similar to the Wb. Baking did not affect β-glucans nor arabinoxylans, favored the release of phenolic compounds from the food matrix and increased antioxidant capacity. Anthocyanins were thermally unstable and decreased after baking. The addition of tartaric acid improved their retention. Purple barley offers new interesting avenues to meet the demand for healthy products.

V.5. CRediT authorship contribution statement

Mariona Martínez-Subirà: Investigation, Writing - original draft. M. Paz Romero: Methodology, Validation, Supervision. Eva Puig: Investigation. Alba Macià: Investigation, Formal analysis. Ignacio Romagosa: Formal analysis, Resources, Funding acquisition, Project administration. Marian Moralejo: Conceptualization, Writing - original draft, Writing - review & editing, Supervision, Funding acquisition, Project administration.

V.6. Declaration of competing interest

None.

V.7. Acknowledgments

We thank Dr. Ana Ferrer (University of Zaragoza) for her support with the eGI measurements.

V.8. Funding

This work was funded by project AGL 2015-69435-C3-1 from the Spanish Ministry of Economy and Competitiveness. Mariona Martínez-Subirà was supported by a pre-doctoral fellowship (BES-2016-078,654/ AGL 2015-69435-C3-1).

V.9. Supplementary information

Table V.S.1.Anthocyanins profile detected in flour and biscuits.

	Flours			Biscuits			Biscuits with tartaric acid (0.5 g /100 g sample)		
	B	**30B**	**30E**	**Bb**	**30Bb**	**30Eb**	**Bb**	**30Bb**	**30Eb**
Antocyanins	**µg /g flour**			**µg /g biscuit**			**µg /g biscuit**		
Pelargonidin glucoside	0.565 ^{b}	0.141 ^{c}	0.876 ^{a}	0.503 ^{b}	0.240 ^{c}	0.602 ^{a}	0.319 ^{a}	0.080 ^{b}	0.350 ^{a}
Pelargonidin acetylglucoside	0.008 ^{b}	-	0.018 ^{a}	0.021	-	-	0.092	-	-
Carboxypyrano pelargonidin glu	-	-	0.030	-	-	0.015	-	-	0.020
Pelargonidin malonylglucoside	2.365 ^{b}	0.739 ^{c}	3.867 ^{a}	0.139 ^{a}	0.016 ^{b}	0.169 ^{a}	0.824 ^{a}	0.170 ^{b}	0.808 ^{a}
Pelargonidin dimalonylglucoside	1.537 ^{b}	0.423 ^{c}	2.244 ^{a}	0.042 ^{a}	-	0.011 ^{a}	0.302 ^{a}	0.065 ^{b}	0.326 ^{a}
Total pelargonidins	**4.475** ^{b}	**1.303** ^{c}	**7.034** ^{a}	**0.704** ^{a}	**0.257** ^{b}	**0.796** ^{a}	**1.537** ^{a}	**0.315** ^{b}	**1.504** ^{a}
Cyanidin glucoside	13.180 ^{b}	3.881 ^{c}	20.327 ^{a}	8.695 ^{b}	3.862 ^{c}	10.804 ^{a}	6.107 ^{a}	1.617 ^{b}	6.616 ^{a}
Cyanidin acetylglucoside	0.158 ^{a}	0.030 ^{b}	0.138 ^{a}	0.321 ^{a}	0.065 ^{b}	0.356 ^{a}	1.656 ^{a}	0.400 ^{b}	1.548 ^{a}
Cyanidin malonylglucoside	0.079 ^{b}	0.030 ^{c}	0.124 ^{a}	0.012 ^{a}	-	0.011 ^{a}	0.025 ^{b}	-	0.037 ^{a}
Cyanidin dimalonylglucoside	16.750 ^{b}	8.678 ^{c}	44.836 ^{a}	0.933 ^{a}	0.111 ^{a}	0.320 ^{a}	4.985 ^{a}	1.157 ^{b}	5.192 ^{a}
Total cyanidins	**30.166** ^{b}	**12.620** ^{c}	**65.425** ^{a}	**9.961** ^{a}	**4.038** ^{b}	**11.491** ^{a}	**12.773** ^{a}	**3.174** ^{b}	**13.393** ^{a}
Peonidin acetylglucoside	-	-	0.024	-	-	0.044	-	0.050 ^{b}	0.237 ^{a}
Peonidin malonylglucoside	0.027 ^{b}	-	0.040 ^{a}	-	-	-	-	-	-
Peonidin dimalonylglucoside	0.013	-	-	-	-	-	0.002	-	-
Total peonidins	**0.040** ^{b}	-	**0.064** ^{a}	-	-	**0.044**	**0.002** ^{b}	**0.050** ^{b}	**0.237** ^{a}
Delphinidin glucoside	0.233 ^{b}	0.071 ^{c}	0.312 ^{a}	0.195 ^{a}	0.069 ^{b}	0.171 ^{a}	0.197 ^{a}	0.084 ^{a}	0.185 ^{a}
Delphinidin acetylglucoside	-	-	-	0.026	-	-	0.063	-	-
Delphinidin malonylglucoside	1.793 ^{b}	0.562 ^{c}	2.799 ^{a}	0.179 ^{ab}	0.013 ^{b}	0.205 ^{a}	0.545 ^{b}	0.121 ^{c}	0.733 ^{a}
Delphinidin dimalonylglucoside	0.097 ^{a}	-	0.164 ^{a}	-	-	-	-	-	-
Total delphinidins	**2.123** ^{b}	**0.633** ^{c}	**3.275** ^{a}	**0.400** ^{a}	**0.082** ^{b}	**0.375** ^{a}	**0.805** ^{a}	**0.206** ^{b}	**0.918** ^{a}
Petunidin malonylglucoside	0.090 ^{b}	0.011 ^{c}	0.143 ^{a}	0.014	-	-	0.025 ^{a}	-	0.027 ^{a}
Petunidin rutinoside	0.036 ^{a}	0.022 ^{a}	0.038 ^{a}	0.018 ^{a}	0.009 ^{a}	0.013 ^{a}	0.014 ^{a}	0.010 ^{a}	0.021 ^{a}
Petunidin hexoside hexoside	0.015 ^{a}	-	0.020 ^{a}	0.014 ^{a}	-	0.022 ^{a}	0.007 ^{a}	-	0.008 ^{a}
Total petunidins	**0.142** ^{b}	**0.032** ^{c}	**0.202** ^{a}	**0.046** ^{a}	**0.009** ^{a}	**0.035** ^{a}	**0.046** ^{a}	**0.010** ^{b}	**0.055** ^{a}
Malvidin malonylglucoside	0.249 ^{a}	0.063 ^{b}	0.205 ^{a}	-	-	-	0.056 ^{a}	0.012 ^{b}	0.028 ^{ab}
Malvidin hexoside hexoside	0.014 ^{a}	-	0.013 ^{a}	0.017 ^{a}	-	0.018 ^{a}	0.006 ^{a}	-	0.005 ^{a}
Malvidin dimalonylglucoside	0.006	-	-	0.007	-	-	0.003	-	-
Total malvidins	**0.269** ^{a}	**0.063** ^{b}	**0.218** ^{a}	**0.024** ^{a}	-	**0.018** ^{a}	**0.065** ^{a}	**0.012** ^{b}	**0.033** ^{ab}
Total anthocyanins	**37.215** ^{b}	**14.652** ^{c}	**76.218** ^{a}	**11.135** ^{a}	**4.386** ^{b}	**12.759** ^{a}	**15.228** ^{a}	**3.765** ^{b}	**16.141** ^{a}

Values as mean of three replicates. For each type of product, flours and biscuits, values within a row followed by different letters indicate significant differences according to Tukey-Kramer's HSD (0.05). - not detected (< LOD).

V.10. References

AACC (2008). AACC international method 10-52.02. Baking quality of cookie flour micro method 1-5. Food Chem. 51, 2174-2180.

Adom KK, Liu RH (2002) Antioxidant activity of grains. J. Agric. Food Chem. 50(21): 6182-6187.

Atkinson FS, Foster-Powell K, Brand-Miller JC (2008) International tables of glycemic index and glycemic load values. Diabetes Care 31:2281-2283.

Baik BK, Ullrich SE (2008) Barley for food: Characteristics, improvement, and renewed interest. J. Cereal Sci. 48:233-242.

Bashir KM, Choi JS (2017) Clinical and physiological perspectives of β-glucans: The past, present, and future. Int. J. Mol. Sci. 18(9):1906.

Blandino M, Locatelli M, Sovrani V, Coïsson JD, Luca Rolle F, Travaglia F, Giacosa S, Bordiga M, Scarpino V, Reyneri A, Arlorio M (2015). Progressive Pearling of Barley Kernel: Chemical characterization of pearling fractions and effect of their inclusion on the nutritional and technological properties of wheat bread. J. Agric. Food Chem. 63(25):5875-5884.

Brennan CS, Tudorica CM (2008) Evaluation of potential mechanisms by which dietary fiber additions reduce the predicted glycaemic index of fresh pastas. Int. J. Food Sci. Technol. 43:2151-2162.

Calinoiu LF, Vodnar DC (2018) Whole grains and phenolic acids: A review on bioactivity, functionality, health benefits and bioavailability. Nutrients 10(11):1615.

Chaaban H, Ioannou I, Chebil L, Slimane M, Gérardin C, Paris C, Charbonnel C, Chekir L, Ghoul M (2016) Effect of heat processing on thermal stability and antioxidant activity of six flavonoids. J. Food Process. Preserv. 41(5): e13203.

De Graaff P, Govers C, Wichers HJ, Debets R (2018) Consumption of β-glucans to spice up T cell treatment of tumors: A review. Expert Opin. Biol. Ther. 18(10):1023-1040.

EFSA (2011). Scientific opinion on the substantiation of health claims related to β-glucans from oats and barley and maintenance of normal blood LDL-cholesterol concentrations (ID 1236, 1299), increase in satiety leading to a reduction in energy intake (ID 851, 852), reduction of post-prandial glycaemic responses (ID 821, 824), and "digestive function" (ID 850) pursuant to Article 13(1) of Regulation (EC) No 1924/ 2006. EFSA Journal, 9(6), 2207.

Fadel A, Mahmoud A, Ashworth J, Li W, Ng YL, Plunkett A (2018) Health-related effects and improving extractability of cereal arabinoxylans. Int. J. Biol. Macromol. 19:819-831.

Fardet A, Leenhardt F, Lioger D, Scalbert A, Rémésy C (2006) Parameters controlling the glycaemic response to breads. Nutr. Res. Rev. 19(1):18-25.

Fardet A, Rock E, Rémésy C (2008) Is the in vitro antioxidant potential of whole-grain cereals and cereal products well reflected in vivo? J. Cereal Sci. 48(2):258-276.

FDA (2008). Food labeling: Health claims; soluble dietary fiber from certain foods and coronary heart disease. [Docket No. FDA-2008-P0090].

Goñi I, García-AIonso A, Saura-Calixto F (1997) A starch hydrolysis procedure to estimate glycemic index. Nutr. Res. 17:427-437.

Granfeldt Y (1994) Foods factors affecting metabolic responses to cereal products. Dept. of Applied Nutrition and Food Chemistry. Lund University.

Hassan AS, Houston K, Lahnstein J, Shirley N, Schwerdt JG, Gidley MJ, Waugh R, Little A, Burton RA (2017) A Genome Wide Association Study of arabinoxylan content in 2-row spring barley grain. PloS One 12:1-19.

Hong F, Yan J, Baran JT, Allendorf DJ, Hansen RD, Ostroff GR, Xing PX, Cheung N-KV, Ross GD (2004) Mechanism by which orally administered β-1, 3-glucans enhance the tumoricidal activity of antitumor monoclonal antibodies in murine tumor models. J. Immunol. 173(2):797-806.

Huang D, Ou B, Hampsch-Woodill M, Flanagan JA, Prior RL (2002) High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96 well format. J. Agric. Food Chem. 50:4437-4444.

Idehen E, Tang Y, Sang S (2017) Bioactive phytochemicals in barley. J Food Drug Anal. 25:148-161.

Izydorczyk MS, Dexter JE (2008) Barley β-glucans and arabinoxylans: Molecular structure, physicochemical properties, and uses in food products-a Review. Food Res. Int. 41:850-868.

Kahkonen MP, Heinonen M (2003) Antioxidant activity of anthocyanins and their aglycons. J. Agric. Food Chem. 51:628-633.

Koddami A, Wilkes MA, Roberts TH (2013) Techniques for analysis of plant phenolic compounds. Molecules, 19;18(2):2328-2375.

Lee C, Han D, Kim B, Back N, Baik BK (2013) Antioxidant and anti-hypertensive activity of anthocyanin-rich extracts from hulless pigmented barley cultivars. Int. J. Food Sci. Technol. 48:984-991.

Malunga LN, Beta T (2015) Antioxidant capacity of water-extractable arabinoxylan from commercial barley, wheat, and wheat fractions. Cereal Chem. 92:29-36.

Martínez M, Motilva MJ, López de las Hazas MC, Romero MP, Vaculova K, Ludwig IA (2018) Phytochemical composition and β-glucan content of barley genotypes from two different geographic origins for human health food production. Food Chem. 245:61-70.

Mosele JI, Motilva MJ, Ludwig IA (2018) Beta-Glucan and Phenolic compounds: Their concentration and behavior during in vitro gastrointestinal digestion and colonic fermentation of different Barley-Based food products. J. Agric. Food Chem. 66:8966-8975.

Pasqualone A, Bianco AM, Paradiso VM, Summo C, Gambacorta G, Caponio F, Blanco A (2015). Production and characterization of functional biscuits obtained from purple wheat. Food Chemistry, 180:64-70.

Regulation EC No 1924/2006 on nutrition and health claims made on foods (2006). European Community (EC) No 1924/2006 of the European Parliament and of the Council of 20 December 2006 on nutrition and health claims made on foods. Official Journal of the European Union, 9-25.

Sajilata MG, Singhal RS, Kulkarni PR (2006) Resistant starch-A review. Food Sci. Food Saf. 5:1-17.

Sharma P, Gujral HS (2014) Cookie making behavior of wheat-barley flour blends and effects on antioxidant properties. LWT-Food Sci. Technol. 55:301-307.

Slavin M, Lu Y, Kaplan N, Yu L (2013) Effects of baking on cyanidin-3-glucoside content and antioxidant properties of black and yellow soybean crackers. Food Chem. 14(2):1166-1174.

Sui X, Yi Yap P, Zhou W (2015) Anthocyanins during baking: Their degradation kinetics and impacts on color and antioxidant capacity of bread. Food Bioproc. Tech, 8: 983-994.

Trogh I, Courtin CM, Andersson AM, Aman P, Sorenson JF, Delcour JA (2004) The combined use of hulless barley flour and xylanase as a strategy for wheat/hulless barley flour breads with increased arabinoxylan and (1→3,1→4)-β-D-glucan levels. J. Cereal Sci. 40:257-267.

Vasanthan T, Gaosong J, Yeung J, Li J (2002) Dietary fiber profile of barley flour as affected by extrusion cooking. Food Chem. 77(1):35-40.

Wani AA, Kumar P (2016) Effect of extrusion on the nutritional, antioxidant and microstructural characteristics of nutritionally enriched snacks. J. Food Process. Preserv. 40(2):166-173.

Wu X, Beecher GR, Holden J M, Haytowitz DB, Gebhardt SE, Prior RL (2004) Lipophilic and hydrophilic antioxidant capacities of common foods in the United States. J. Agric. Food Chem. 52-4026-4037.

Zhang XW, Jiang QT, Wei YM, Liu C (2017) Inheritance analysis and mapping of quantitative trait loci (QTL) controlling individual anthocyanin compounds in purple barley (*Hordeum vulgare* L.) grains. PloS One, 12(8), Article e0183704.

Chapter VI. General discussion

VI.1. Sensitivity of bioactive compounds to heat stress

Barley is the largest crop by planted area in Spain and other semiarid Mediterranean areas suffering temperature stress at the end of the season. Climate model projections suggest that higher temperatures will become common in most regions where cereals are grown (IPCC, 2013). High temperature during the grain fill development stage is a major limiting factor for grain yield and quality (Jenner, 1994). In addition, the magnitude of the effect depends on the genotype, time of exposure and duration of the stress (Savin and Molina-Cano, 2002). The reduction in grain size is the main effect of high temperature after anthesis, with grain weight losses of between 5% and 30%. This same trend was observed in Chapter II of this thesis, where reduction in grain weight was also reflected in the grain size and plumpness, these being lower under heat stress.

While the harmful effects of high temperatures on crop yield and grain quality are well documented in the literature (Savin and Nicolas, 1996; Savin et al., 1996; Wallwork et al., 1998; Passarella et al., 2002), publications about the effect of heat stress on the bioactive compounds in barley are very limited and some of the results are inconsistent. In this context, in Chapter II we determined if the final bioactive contents were sensitive to a heat stress period from the middle of grain filling to physiological maturity or if, on the contrary, sensitivity only depended on the genotype. Our results showed that heat stress indirectly affected the dietary fibre content and some specific compounds, such as ferulic acids, since their final increases in grains subjected to heat stress were attributed to a concentration effect of these compounds in lighter stressed grains. However, heat stress also had direct significant impacts on other phenolic compounds, especially coumaric acids and flavan-3-ols, increasing their final content in stressed grains. Therefore, grains grown under warmer conditions showed improved health properties, since their β-glucan and arabinoxylan concentrations were equal to or greater than in non-stressed conditions despite the low flour yields due to the thinner grains. In addition, these grains had higher concentrations of some specific phenolic compounds (up to 20% depending on the genotype). Thus, they also had a higher antioxidant capacity by up to 30% (again genotypic dependent). This could have occurred to protect the plant cells from heat-induced oxidative damage (Sharma et al., 2019).

We do not know if, besides having significant direct and indirect impacts on the final content of the main bioactive compounds, heat stress could cause structural modifications of these compounds in the grains. Such alterations have been reported in wheat, where environmental effects caused minor effects on the structure of β-glucan and arabinoxylan (Rakszegi et al., 2014). Therefore, future research would be necessary to determine whether the structure of some of these bioactive compounds is affected by heat stress as this can influence the final quality of the barley-based product.

High temperature stress at the end of season, frequent in Mediterranean areas, produces smaller and lighter grains, not suitable for malting. However, this stress could be favourable in the context of food barley, as some of its contents of bioactive compounds and antioxidant capacity will be increased, regardless of the smaller size grains. Furthermore, if a market develops for food barley, late sowing in the most important producing areas could be a possibility if a potential premium can compensate for the associated decrease in crop yield. In this context, from the breeding point of view, we could also suggest regular sowing dates for later flowering genotypes so the grain filling takes place under warmer temperatures.

Association between variables

The bioactive compound contents of barley are strongly influenced by genetic diversity and growing conditions. The association between the genotypes analysed (Annapurna, Tamalpais Hispanic and Hindukusch), environments (control vs. heat stress conditions), bioactive compounds and antioxidant capacity is presented graphically in Figure VI.1. These results were obtained by principal components analysis (PCA) carried out on the across-year average bioactive profile of the grains from the four genotypes grown under controlled and heat stress induced conditions during grain filling. The two main axes of the PCA together explain more than 80% of the total variability. The first axis is particularly related to the antioxidant capacity (AC) and its components. The second axis is associated with the contrast between the flavan-3-ols and β-glucans *vs*. coumaric acid concentrations as determined by the bioactive profile of the Annapurna and Tamalpais genotypes compared to the Hispanic and Hindukusch. As expected, antioxidant capacity is associated with free and bound ferulic acids, which were the quantitatively more important phenolic acids, and with anthocyanins. Arabinoxylans is correlated with ferulic acid due to their structures being mainly linked in the outer layers of the barley grain (Izydorczyk and Dexter, 2008). The effect of the induced stress is seen in Figure VI.1. Differential behaviour of the four genotypes associated with the heat stress induced was observed. The antioxidant capacity of the Annapurna, Hispanic and Tamalpais genotypes increased due to induced stress. Their PCA1 values increased, while the Hindukusch reacted in the opposite direction, as its PCA1 score decreased, probably due to a reduction of anthocyanins associated with the polyethylene film used to cover the plots.

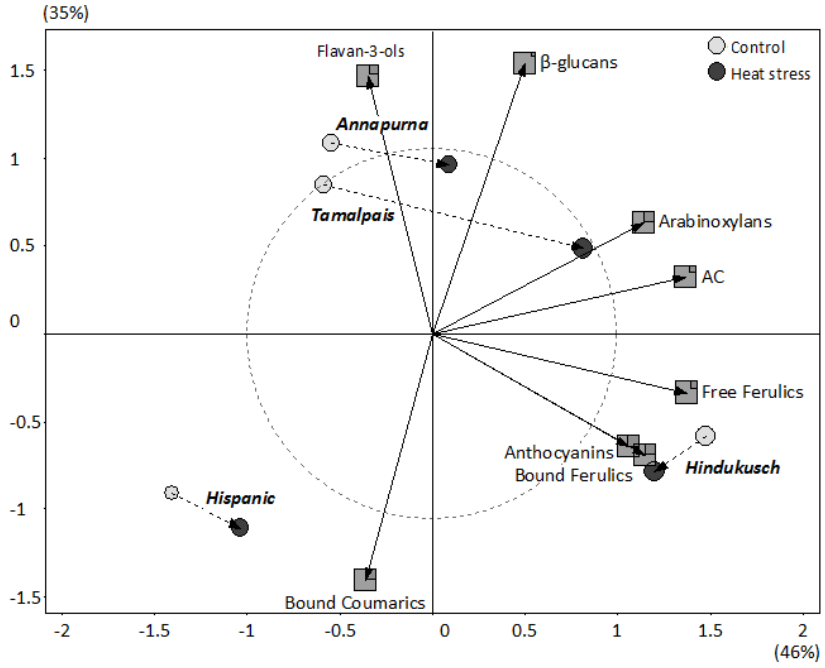


Figure VI.1. Principal Component analysis of dietary fibre, phenolic compounds and antioxidant capacity in the grain of four barley genotypes grown under control (light circles) and heat stress (dark circles) during grain filling. The darker square within the lighter one for each bioactive compound is proportional to the

unexplained variance. The size of the circles for each genotype under control or induced stress is proportional to their antioxidant capacity.

VI.2. Accumulation and deposition patterns of barley grain bioactive compounds

Agronomic and environmental conditions during the barley growing cycle strongly influence the grain yield and composition (Zhou et al., 2020). As mentioned above, barley grown in the toughest environments or in semiarid areas is not suitable for malting, as the grain does not achieve minimum requirements. On the contrary, we have found that food barley may profit from high temperatures at the end of the season increasing its contents of bioactive compounds. However, determination of the dynamics of the accumulation of these bioactive compounds is a crucial aspect for enhancing the nutritional values of barley in order to provide health benefits. Chapter III of this thesis focuses on the deposition pattern of bioactive compounds and antioxidant capacity in grains regularly harvested from a number of individual spikes marked at anthesis from four barley genotypes, under two temperatures under well irrigated conditions. Four genotypes, key parents in our food barley breeding program, were selected. They differed not only in the grain characteristics (number of rows, presence/absence of hulls, type of starch, grain colour) but also in an array of bioactive compounds, potentially susceptible to heat stress from 15 days after heading to physiological maturity, as shown in Figure VI.1.

The results showed that the maximum content of dietary fibre in barley grains is obtained at or near physiological maturity, as β-glucans and arabinoxylans were synthesized all through grain filling at the same rate as dry matter. On the contrary, our results suggest early harvesting of non-mature grains to maximize antioxidant capacity. Phenolic compounds were synthesized in early stages and decreased toward grain maturation, implying a drop in antioxidant capacity during grain development, since these bioactive compounds make a significant contribution to the total antioxidant capacity, as described in the literature (Zhu et al., 2015; Suriano et al., 2018). The reduction of phenolic compounds during grain maturation could be due to four possible aspects: (I) An early activity of the enzyme that catalyses the conversion of phenylalanine to transcinnamic acid during the biosynthesis of phenolic compounds (McCallum and Walker, 1991). (II) A dilution effect due to the accumulation of starch in the growing endosperm (Knievel et al., 2009). (III) A compromise of carbohydrate availability between starch synthesis and phenolic compound biosynthesis during the grain development (Schulman et al., 2000). (IV) A decrease in physiological processes, such as photosynthesis or oxidative metabolism, during the grain dehydration process (Özkaya et al., 2018). If a market develops for barley food ingredients, early harvesting of immature grain should be considered to maximize antioxidant capacity.

The deposition rate of bound phenols (major phenolic compounds in barley) was more sensitive to genotypic diversity than that for free phenols. Unlike the non-coloured genotypes, the purple variety showed a slight increase in bound phenolic compounds towards the end of grain filling. The bound phenol accumulation could be attributed to conversion between fractions (from free to bound) during grain filling. André et al. (2009) suggested that the content of a phenolic compound is regulated by the rates of biosynthesis and degradation. In other words, high levels of a specific phenolic compound could be attributed to the degradation of another phenolic compound. Bustos et al. (2012) observed that the high phenolic compound contents in coloured wheat grains could be attributed to the reduction in anthocyanins in the final phase of grain filling. However, degradation

of phenolic compounds and conversion between fractions (from free to bound) in barley requires future studies. These results suggest that agronomic treatments such as harvest time, to increase the contents of phenolic compounds and ultimately the antioxidant capacity of the grain, depend on genetic diversity. For instance, for a coloured genotype with high anthocyanin content, harvesting in the late stages of grain filling may be more effective than at an earlier time.

VI.3 Spatial distribution of bioactive compounds in food barley grain to identify the most appropriate barley fraction for specific products.

Despite the fact that barley has bioactive compounds with recognized health properties, the amount of barley used for food is still very small. Moreover, most of the barley used for food production is of a type in which the grains are covered with a very resistant inedible husk. This outer husk that covers the bran layer must be removed before the grain can be used as food (FDA, 2006). The husk of many barley varieties is strongly adhered to the pericarp, making it difficult to de-husk, which is why barley usually undergoes pearling processes before it can be used in the food industry. The pearling process is an abrasive technique that gradually removes the outer layers of the grain and enables polished grain and by-products to be obtained. The processing that barley must undergo before human consumption affects its composition and physicochemical properties, compromising the health-promoting bioactive components in the grain (Sharma and Kotari, 2017). The husk represents about 10-13% of the dry grain weight, but the current commercial pearling method involves removing more than the husk, about 45% of the grain weight to produce “quick-cook” grains (Tricase et al., 2018). So, pearl barley should not be considered a whole grain because part of the bran and germ layer has been removed.

In Chapter IV the pearling process was used to compare the spatial distribution of ash, proteins, bioactive compounds and antioxidant capacity in the grain of three barley genotypes differing in their husk contents, in order to identify the best grain fractions for use as functional ingredients. Our results highlight that the husks are a rich source of arabinoxylans and some specific major phenolic compounds, resulting in a high antioxidant capacity, similar to that of the outermost layers. Although current regulations require removal of the inedible husks from hulled barley for use as food (FDA, 2006), whole hulled barley grain or the external husks should be considered as a valuable material for animal feed due to their high contents of bioactive compounds. The outermost layers of hull-less or de-husked barley are the most valuable source of phenolic compounds, antioxidant capacity, arabinoxylans and tocols. Therefore, for hulled grains intended for food, it would be interesting to apply commercial techniques such as husk peeling or pearling of only 10% of the grain weight. This way, the polished grain would not only be suitable for food, but would also partially maintain the layers with a high content of bioactive compounds, increasing its potential beneficial effects. The intermediate layers provide high levels of proteins, β-glucans, tocopherols and some phenolic compounds, such as catechins and hydroxybenzoic acid. Finally, the endosperm has the highest β-glucan contents and a relatively high presence of catechins and hydroxybenzoic acid.

Hence, pearling allows a favourable separation of fractions enriched in specific compounds that can be used as functional ingredients. An enrichment of antioxidant compounds has been described in pearl barley fractions (Irakli et al., 2020), improving the nutritional value of wheat-based products such as biscuits, pasta and bread (Marconi et al., 2000; Sharma and Gujral, 2014; Blandino et al., 2015). Although pearl barley is not considered whole grain because the bran layer and germ are

removed, the polished grain is used in the preparation of many traditional dishes and is also used to produce barley tea, miso, and rice diluent in the Japanese and Korean markets. Additionally, barley flour made from polished grain has been used to produce bread, cakes, muffins, cookies, noodles, and snacks (Tricase et al., 2018).

VI.4. Use of bioactive compounds as functional ingredients

The different composition of the ingredients derived from the pearling process offers the possibility of having several fractions available to select the best ones to produce enriched foods in bioactive compounds. This is as shown in chapter V, where biscuits, with different proportions of whole purple barley flour and selected pearling fractions with different proportions of refined and whole-wheat flour, were made to explore their functional properties. As far as we know, this was the first time hull-less purple barley grain has been used for the production of functional food.

Substituting 30% refined wheat flour with whole barley flour resulted in biscuits with a tenfold increase in the concentration of β-glucans. The addition of 30% of the external fraction increased the arabinoxylan contents by four to reach similar levels as those for whole-wheat biscuits. Both types of biscuit considerably increased the dietary fibre content of wheat biscuits considered to be a “source of fibre”, since they contained more than 3 g fibre/100 g food (Regulation EC No 1924/2006). Besides, the addition of 30% of the external barley fraction or whole barley flour also increased the phenolic compound contents and the antioxidant capacity, due to the low levels of these antioxidants compounds in refined and whole-wheat flours.

The main challenge of adding barley flours or fractions to bakery products is finding new processing strategies to incorporate more bioactive compounds (Blandino et al., 2015). The thermal treatments may have a positive or negative influence on bioactive compounds during the baking process. As observed in the present study, baking did not affect β-glucans or arabinoxylans, but affected individual phenolic compounds differently and increased the antioxidant capacity. The total free phenolic compounds remained stable due to the compensation between the increase in the phenolic acids, aldehydes and flavone glycosides and the decrease in most flavan-3-ols. The bound phenolic compounds, especially coumaric and ferulic acids, increased during baking in all biscuits. The increase in specific phenolic compounds seems to be a balance between the decarboxylation of phenolic acids by exposure to high temperatures (Wani and Kumar, 2016) and their release from the fibre. In addition, some phenolic compounds may not be fully recovered during the extraction process and thus the total components may be underestimated (Adom and Liu, 2002; Koddami et al., 2013). In our case, the antioxidant capacity increased significantly after baking, as did the phenolic compounds. This can be attributed to the above-mentioned release of phenolic acids from the food matrix, the formation of some products from the Maillard reaction, or the presence of other products newly formed after thermal treatment exhibiting antioxidant capacity sometimes superior to that of native molecules (Chaaban et al., 2016). Anthocyanins were thermally unstable and decreased after baking. Thermal degradation begins with the hydrolysis of the sugar moieties, which are then degraded to chalcones. The subsequent breakdown of chalcones results in the formation of phenolic acids and carboxyaldehydes (Sui et al., 2015). However, it has been suggested that the degradation products retain antioxidant properties and hence, thermal degradation may not have a significant impact on the antioxidant capacity of the final product (Slavin et al., 2013). The addition of tartaric acid improved the anthocyanin retention and preserved the pinkish colour

in the biscuits, rather than an unappetizing greenish appearance. However, future research is needed to evaluate the effect of other acidulates and their concentrations on the anthocyanin content of barley biscuit formulations. Overall, hull-less and coloured-grain barley is a valuable source of bioactive compounds to be considered in bakery, taking into account the possible introduction of strategies to mitigate undesirable effects during processing, for example the addition of acidulates to the dough to reduce the loss of anthocyanins.

Beyond the growing interest in barley bioactive compounds for food, it is also necessary to improve the health of consumers due to the high incidence of such food-related problems as obesity, diabetes, high blood pressure, heart disease and some types of cancers. One of our biscuit exclusively made from purple barley provided more than 0.75 g of β-glucans per unit, which is the limit defined by EFSA for labelling (EFSA, 2011). Moreover, one serving of four barley biscuits would satisfy the 3 g of β-glucans per day goal to support the claim of reducing blood cholesterol and the risk of heart disease (EFSA, 2011). Additionally, barley biscuits also fulfilled the claim of "high in fibre" for products containing at least 6 g fibre/100 g food, due to high β-glucan and arabinoxylan contents (Regulation EC No 1924/2006). Biscuits containing purple barley ingredients had a lower estimated glycaemic index than conventional wheat-based biscuits. This could be attributed to their higher dietary fibre content (Brennan and Tudorica, 2008) and the higher amylose/amylopectin ratio since amylose is more resistant to digestive alpha-amylases (Sajilata et al., 2006), resulting in a reduced glycaemic response.

The results found in the present study showed that barley offers interesting new avenues to meet the demand for healthy products. However, the most important current challenge is to introduce barley into the market, and this can be most easily achieved by means of derivatives of bread or pastries, pasta or breakfast cereals. Sensory quality can be an obstacle for using barley as a healthy food crop and for restoring barley's status in the human diet (Baik, 2016). The results in chapter V show that the inclusion of a percentage of barley flour in wheat-based biscuits caused no reductions in their physical properties, while the use of barley flour or external fraction presented sensory characteristics similar to those of a whole-wheat biscuit widely accepted by consumers. Other authors reported no reductions in processing, product, or sensory quality with the addition of up to 30% barley flour to wheat-based products that required various degrees of gluten development (bread, noodles, and pasta products), not even with the inclusion of higher proportions of barley flour in products that required little or no gluten development (cupcakes and cookies) (Baik, 2016). Therefore, partially replacing wheat flour with barley flour in food formulation seems to be a good way to introduce barley into consumers' diets without compromising the sensory quality of the product.

VI.5. References

Adom KK, Liu RH (2002) Antioxidant activity of grains. J. Agric. Food Chem. 50(21): 6182-6187.

André CM, Schafleitner R, Legay S, Lefèvre I, Alvarado-Aliaga CA, Nomberto G, Hoffmann L, Hausman J-F, Larondelle Y, Evers D (2009). Gene expression changes related to the production of phenolic compounds in potato tubers grown under drought stress. Phytochem. 70:1107-1116.

Baik B-K (2016) Current and potential barley grain food products. Cereal Foods World 61:188–196.

Blandino M, Locatelli M, Sovrani V, Coïsson JD, Rolle L, Travaglia F, Giacosa S, Bordiga M, Scarpino V, Reyneri A, Arlorio M (2015) Progressive Pearling of Barley Kernel: Chemical Characterization of Pearling Fractions and Effect of Their Inclusion on the Nutritional and Technological Properties of Wheat Bread. J. Agric. Food Chem. 63:5875–5884.

Brennan CS, Tudorica CM (2008) Evaluation of potential mechanisms by which dietary fibre additions reduce the predicted glycaemic index of fresh pastas. Int. J. Food Sci. Technol. 43:2151-2162.

Bustos DV, Riegel R, Calderini DF (2012) Anthocyanin content of grains in purple wheat is affected by grain position, assimilate availability and agronomic management. J. Cereal Sci. 55:257–264.

Chaaban H, Ioannou I, Chebil L, Slimane M, Gérardin C, Paris C, Charbonnel C, Chekir L, Ghoul M (2016) Effect of heat processing on thermal stability and antioxidant activity of six flavonoids. J. Food Process. Preserv. 41(5): e13203.

EFSA (2011). Scientific opinion on the substantiation of health claims related to β-glucans from oats and barley and maintenance of normal blood LDL-cholesterol concentrations (ID 1236, 1299), increase in satiety leading to a reduction in energy intake (ID 851, 852), reduction of post-prandial glycaemic responses (ID 821, 824), and “digestive function” (ID 850) pursuant to Article 13(1) of Regulation (EC) No 1924/ 2006. EFSA Journal, 9(6), 2207. Available: http://www.efsa.europa.eu/en/efsajournal/pub/2207 [5 January 2021].

FDA (2006). Draft Guidance for Industry and FDA Staff: Whole Grain Label Statements (Docket No. FDA-2006-D-0298). Issued by: Center for Food Safety and Applied Nutrition. Available: https://www.regulations.gov/docket?D=FDA-2006-D-0298 [21 January 2021].

IPCC, 2013. Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change; TF Stocker, D Qin, G-K Plattner, MMB Tignor, SK Allen, J Boschung, A Nauels, Y Xia, V Bex, PM Midgley, (Ed).; Cambridge University Press: Cambridge, UK; New York, NY, USA, 2013; p. 1535.

Irakli M, Lazaridou A, Mylonas I, Biliaderis CG (2020) Bioactive Components and Antioxidant Activity Distribution in Pearling Fractions of Different Greek Barley Cultivars. Foods 9:783.

Izydorczyk MS, Dexter JE (2008) Barley: β-glucans and arabinoxylans: Molecular structure, physicochemical properties, and uses in food products - a Review. Food Res. Int. 41: 850-868.

Jenner CF (1994). Starch synthesis in the kernel of wheat under high temperature conditions. Funct. Plant Biol. 21:791–806.

Knievel DC, Abdel-Aal ESM, Rabalski I, Nakamura T, Hucl P (2009) Grain color development and the inheritance of high anthocyanin blue aleurone and purple pericarp in spring wheat (*Triticum aestivum* L.). J. Cereal Sci. 50:113–120.

Koddami A, Wilkes MA, Roberts TH (2013) Techniques for analysis of plant phenolic compounds. Molecules, 19;18(2):2328-2375.

Marconi E, Graziano M, Cubadda R (2000) Composition and Utilization of Barley Pearling By-Products for Making Functional Pastas Rich in Dietary Fiber and β-Glucans. Cereal Chem. J. 77:133–139.

McCallum JA, Walker JR (1991) Phenolic biosynthesis during grain development in wheat (*Triticum aestivum* L.) III. Changes in hydroxycinnamic acids during grain development. J. Cereal Sci. 13: 161–172.

Özkaya B, Turksoy S, Özkaya H, Baumgartner B, Özkeser İ, Köksel H (2018) Changes in the functional constituents and phytic acid contents of firiks produced from wheats at different maturation stages. Food Chem. 246:150–155.

Passarella VS, Savin R and Slafer GA (2002) Grain weight and malting quality in barley as affected by brief periods of increased spike temperature under field conditions. Aust J Agric Res 53: 1–9.

Rakszegi M, Lovegrove A, Balla K, Láng L, Bedő Z, Veisz O and Shewry PR (2014) Effect of heat and drought stress on the structure and composition of arabinoxylan and β-glucan in wheat grain. Carbohydr. Polym. 102;557-568.

Regulation EC No 1924/2006 of the European Parliament and of the Council of 20 December 2006 on nutrition and health claims made on foods. Official Journal of the European Union. Available: https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2006:404:0009:0025:EN:PDF [21 January 2021].

Sajilata MG, Singhal RS, Kulkarni PR (2006) Resistant starch-A review. Food Sci. Food Saf. 5:1-17.

Savin R and Molina-Cano JL (2002). Changes in malting quality and its determinants in response to abiotic stresses. In: GA Slafer, JL Molina-Cano, R Savin, JL Araus and I Romagosa (Ed.) Barley Science. Recent advances from molecular biology to agronomy of yield and quality. Food Product Press, New York, USA, pp.523-550.

Savin R and Nicolas ME (1996) Effects of short periods of drought and high temperature on grain growth and starch accumulation of two malting barley cultivars. Aust J Plant Physiology 23: 201–210.

Savin R, Stone PJ, Nicolas ME (1996) Responses of grain growth and malting quality of barley to short periods of high temperature in field studies using portable chambers. Aust. J. Agric. Res. 47: 465-477.

Schulman AH, Runeberg-Roos P, Jääskeläinen M (2000) Grain filling and starch synthesis in barley. In: AK Gupta, N Kaur (Ed.), Carbohydrate Reserves in Plants-Synthesis and Regulation, 1st ed.; Elsevier Science B.V.: Amsterdam, New York The Neth-erlands, 2000; pp. 147-167.

Sharma A, Shahzad B, Rehman A, Bhardwaj R, Landi M and Zheng B (2019) Response of Phenylpropanoid Pathway and the Role of Polyphenols in Plants under Abiotic Stress. Molecules 24: 2452.

Sharma P, Gujral HS (2014) Cookie making behavior of wheat-barley flour blends and effects on antioxidant properties. LWT 55:301–307

Sharma P, Kotari SL (2017) Barley: impact of processing on physico-chemical and thermal properties—a review. Food Rev. Int. 33:359-381.

Slavin M, Lu Y, Kaplan N, Yu L (2013) Effects of baking on cyanidin-3-glucoside content and antioxidant properties of black and yellow soybean crackers. Food Chem. 14(2):1166-1174.

Sui X, Yi Yap P, Zhou W (2015) Anthocyanins during baking: Their degradation kinetics and impacts on color and antioxidant capacity of bread. Food Bioproc. Tech, 8: 983-994.

Suriano S, Iannucci A, Codianni P, Fares C, Russo M, Pecchioni N, Marciello U, Savino M (2018) Phenolic acids profile, nutritional and phytochemical compounds, antioxidant properties in colored barley grown in southern Italy. Int. Food Res. J. 113:221–233.

Tricase C, Amicarelli V, Lamonaca E, Rana RL (2018) Economic analysis of the barley market and related uses. In: Z Tadele (Ed.), Grasses as Food and Feed. IntechOpen, London, UK. pp25-46.

Wallwork MAB, Logue SJ, MacLeod LC and Jenner CF (1998) Effect of high temperature during grain filling on starch synthesis in the developing barley grain. Aust J Plant Physiol 25:173-181.

Wani AA, Kumar P (2016) Effect of extrusion on the nutritional, antioxidant and microstructural characteristics of nutritionally enriched snacks. J. Food Process. Preserv. 40(2):166-173.

Zhou B, Jin Z, Schwarz P and Li Y (2020) Impact of Genotype, Environment, and Malting Conditions on the Antioxidant Activity and Phenolic Content in US Malting Barley. Fermentation 6:48.

Zhu Y, Li T, Fu X, Abbasi AM, Zheng B, Liu RH (2015) Phenolics content, antioxidant and antiproliferative activities of dehulled highland barley (*Hordeum vulgare* L.). J. Funct. Foods. 19:439–450.

Chapter VII. Conclusions

The main conclusions of this Thesis are summarized below:

- Although heat stress produced low flour yields due to thinner grains, grain grown under warmer temperatures had higher phenolic compound contents and antioxidant capacity as well as dietary fibre concentrations equal to or greater than under non-stressed conditions, and thus, enhanced health properties. Hence, final grain quality could, at least partially, be controlled in order to increase the bioactive concentrations in the barley grain by cultivation in growing areas prone to heat stress, such as most Mediterranean regions.

- The maximum level of dietary fibre in barley grains is obtained at or near physiological maturity. On the contrary, phenolic compounds and ultimately antioxidant capacity decreases as the grain develops. So, if a market develops for barley food ingredients, early harvesting of non-mature grain should be considered to maximize antioxidant capacity, taking into account that the rate of deposition of some phenolic compounds could vary between genotypes.

- Whole hull-less or lightly pearled grains as well as their specific pearling fractions, could be used as a source of valuable functional ingredients. The outermost layers of hull-less barley or previously de-husked grains represent the most valuable source of arabinoxylans, phenolic compounds, tocols and antioxidant capacity. The intermediate layers could be considered a rich source of proteins, tocopherols and some phenolic compounds, such as catechins and hydroxybenzoic acid, and the endosperm the largest reservoir of β-glucans.

- The husks and whole hulled barley grains should be considered as a valuable material for animal feed due to their high content of arabinoxylans, some specific major phenolic compounds and high antioxidant capacity in the husks.

- Purple barley offers interesting new avenues to meet the demand for healthy products, since its flour provides higher contents of dietary fibre and bioactive compounds, greater antioxidant capacity and a lower estimated glycaemic index than commercial refined and whole-wheat flours.

- Heat treatment during food processing can affect the bioactive compound contents. In our case, baking did not affect β-glucans or arabinoxylans, favoured the release of phenolic compounds from the food matrix and increased the antioxidant capacity, but decreased the level of anthocyanins due to their thermal instability. However, the addition of tartaric acid improved their retention by acting as a stabilizing agent. So, the addition of an acidulant could be a good strategy to mitigate such undesirable effects as unappetizing colour or loss of some phenolic compounds during the processing of baking barley products.

- The inclusion of 30% barley flour to wheat-based biscuits did not cause reductions in physical properties, while biscuits made only with barley flour presented similar sensory characteristics to those of whole-wheat biscuits widely accepted by consumers.

- Barley grown even in a late heat-stressed area remains a valuable source of bioactive compounds and offers interesting new avenues to meet the demand for healthy diets. Barley-

based products are rarely found on the market. In order to achieve higher barley consumption, it is important to develop various barley food products that offer potential health benefits with favourable sensory qualities. This is the most immediate challenge for the commercial success of food barley.

Annex.

Prediction of bioactive compounds in barley by near-infrared reflectance spectroscopy (NIRS)

Journal of Food Composition and Analysis 97 (2021) 103763

Contents lists available at ScienceDirect

Journal of Food Composition and Analysis

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

Original Research Article

Prediction of bioactive compounds in barley by near-infrared reflectance spectroscopy (NIRS)

Elena Albanell[a, *], Mariona Martínez[b], Massimo De Marchi[c], Carmen L. Manuelian[c, *]

[a] Group of Ruminant Research (G2R), Department of Animal and Food Sciences, Universitat Autònoma de Barcelona (UAB), 08193, Bellaterra, Spain
[b] Food Technology Department, Universitat de Lleida-Agrotecnio Center, Lleida, Alcalde Rovira Roure 191, 25198, Lleida, Spain
[c] Department of Agronomy, Food, Natural Resources, Animals and Environment (DAFNAE), University of Padova, Viale dell'Università 16, 35020, Legnaro, PD, Italy

ARTICLE INFO

Keywords:
Anthocyanin
Arabinoxylan
Barley
β-glucan
Near-infrared
Phenolic compounds

ABSTRACT

Barley grains contain a variable amount of biologically active compounds such as non-starch polysaccharides and phenol compounds. These compounds are important in nutrition due to their significant health benefits and technological role in food. We developed predictive models for β-glucans (BG), arabinoxylans (AX), bound phenols (BP), free phenols (FP), and anthocyanins (AN) based on near-infrared spectroscopy (NIRS) using two different NIRS instruments with different spectral range and spectral steps. Regressions of modified partial least squares (MPLS) and several combinations of scattering correction and derivative treatments were tested. The optimal calibration models generated high coefficients of determination for BG and BP, but not for AN content. The instrument with the highest resolution only gave better results for BG prediction models, and the addition of the visible range did not prove to be ostensibly advantageous to the determination of any of the active compounds of study, not even in the case of AN analysis.

1. Introduction

Barley (*Hordeum vulgare* L.) is one of the most ancient crops and, thanks to domestication, today is one of the major crops in the world in terms of acreage and production, which significantly contributes to the world's food supply (Gupta et al., 2010). It is grown for many uses such as food source for human beings and animals as well as industrial food processing (e.g., malting, brewing, and baking) (Gordon et al., 2019).

Nowadays, there is an increasing trend to provide consumers safe foods based on cereal grains and/or components with high nutritive value to promote their health. Cereal grains contain a variable amount of biologically active compounds such as dietary fiber (e.g., arabinoxylans, β-glucans, cellulose, and lignin), sterols, tocopherols, phenolic compounds, vitamins, and microelements (Bartłomiej et al., 2012; Blakeney and Flinn, 2005). Compared to other grains, barley has a relatively high concentration of (1→3),(1→4)-β-glucans (β-glucan) (BG) and arabinoxylans (AX) which depend on genetic and environmental factors (Gupta et al., 2010). Both BG and AX are associated with cell walls of cereals. Type and composition of these non-starch polysaccharides and phenolic compounds is of increasing importance in the nutrition of both humans and animals due to their significant health benefits and technological role in food. Consumption of cereals containing BG and/or phenolic compounds contributes to a decreased risk of chronic health problems, such as those associated with cardiovascular diseases and diabetes, by reducing blood cholesterol and glucose levels, and it can also contribute in preventing some types of cancer (Gupta et al., 2010; Han et al., 2017; Li et al., 2003).

Conventional analyses to determine bioactive compounds of barley are based on chemical and enzymatic analyses which provide the basis to calculate the accuracy and precision of new methods/analytical tools. However, these conventional methods require highly trained technicians/workforce, are time-consuming and labor-intensive, use several chemical reagents, and destroy the sample during the analysis. As a consequence, the development of a simple, rapid, and cost-effective method for measuring bioactive compounds in barley would be of great benefit for the food industry.

Near-infrared spectroscopy (NIRS) is a technique that collects the reflected light of a sampled material in the near-infrared region of the electromagnetic spectrum and can provide information on the chemical composition of agricultural products. As well as being a rapid analytical method and a relatively cost-effective alternative to conventional analyses, it does not destroy the sample, thus allowing further analysis. The

* Corresponding authors.

E-mail addresses: elena.albanell@uab.cat (E. Albanell), marionamar@pvcf.udl.cat (M. Martínez), massimo.demarchi@unipd.it (M. De Marchi), carmenloreto.manuelianfuste@unipd.it (C.L. Manuelian).

https://doi.org/10.1016/j.jfca.2020.103763
Received 21 July 2020; Received in revised form 15 November 2020; Accepted 12 December 2020
Available online 15 December 2020

use of NIRS for quality control of cereals is well established in the literature (Osborne, 2000). Some authors have shown the possibility to predict BG content in intact barley seeds using different near-infrared instruments, resolution, and wavelength range: De Sá and Palmer (2006) used the wavelength range between 900 1700 nm; Sohn et al. (2008) used three NIRS instruments with different resolutions, one Fourier transform near-infrared (FT-NIR) and two dispersive instruments (8 nm and 10 nm bandpass); Ringsted et al. (2017) used long wavelength NIRS (2260-2380 nm). In naked barley, Schmidt et al. (2009) quantified BG content and compared different types of near-infrared instruments: FT-NIR, NIT (near-infrared transmission), and Vis-NIR (visible and near-infrared; 400-2498 nm, scanning at 2-nm intervals). Moreover, a previous study suggested the feasibility of NIRS to predict AX in a set of grains and pulses (Blakeney and Flinn, 2005), but, to the best of our knowledge, there are no studies that measured AX in barley flour using NIRS. Although the NIRS technique has been successfully applied for the determination of total phenols in sorghum (Alfieri et al., 2019; Dykes et al., 2014), rice (Zhang et al., 2008), and barley grains (Han et al., 2017), studies describing the use of NIRS technique to measure anthocyanins in barley are lacking.

This study aimed to develop NIRS prediction models for rapid measurement of non-starch polysaccharides (BG and AX), phenols compounds (free and bound phenolic acids - FP and BP), and anthocyanins (AN) of barley flour as well as to compare the accuracy of two different NIRS instruments.

2. Material and methods

2.1. Barley samples

A total of 186 barley samples were selected from a previous genetic study to obtain genotypes with high BG and amylose contents and enhanced antioxidant activity. All barley varieties used in the present study were grown in Spain (Semillas Batlle, SA, Bell-lloc d'Urgell, Spain) during 2016, 2017, and 2018 and included hulled and hull-less genotypes. Barley grain samples were stored at room temperature in a desiccator and in the dark until use. Prior to the analysis, barley grain samples were ground in a Foss Cyclotec 1093™ (FOSS, Hillerød, Denmark) mill equipped with a 0.5 mm screen to produce whole grain flour. A sub-sample of each sample flour was used for wet chemical analysis and the remnant flour was used for NIRS analysis. The ground samples were stored at 4 °C until analysis.

2.2. Chemical analysis

Content of BG was determined with the β-glucan (mixed-linkage) from Megazyme (Bray, Ireland). Briefly, 0.2 mL of aqueous ethanol (50 % v/v) and 4 mL of sodium phosphate buffer (20 mmol/L, pH 6.5) were added to 80 mg of whole grain flour. After that, the samples were vortexed, incubated, and tempered at 50 °C. Then, 0.2 mL lichenase enzyme (10U) was added to the samples tube and incubated. After incubation, sodium acetate buffer was added and centrifuged at 1000 × *g*. For each sample, 0.1 mL of the supernatant was transferred to other tubes, and 0.1 mL of β-glucosidase (0.2U) was added. The reaction blanks were also prepared with 0.1 mL of each sample and 0.1 mL of sodium acetate buffer was added. The mixtures were incubated a 50 °C for 50 min and then, glucose oxidase/peroxidase reagent was added and the mixture was incubated at 50 °C for 20 min. After that, the absorbance was read at 510 nm using a spectrophotometer Jenway 6300 (Essex, England). The results were expressed as g/100 g of whole grain flour.

The arabinoxylans content was analyzed with the D-Xylose (Xylan & Arabinoxilan) kit from Megazyme (Bray, Ireland). Fifty mg of each barley flour sample was placed in 50 mL centrifuge tubes and 2.5 mL of HCl 1.3 M were added. Then, the samples were incubated, vortexed, cooled to room temperature, and 2.5 mL of NaOH 1.3 M was added to each. For each sample, 10 μL of the supernatant was transferred to a well on the microplate. Absorbance readings at 340 nm were taken using a Multiscan GO spectrophotometer (Thermo Scientific, Vantaa, Finland). The results were expressed as g/100 g of whole grain flour.

Free phenolic compounds were extracted using 1 mL of the extraction solution (79.5 % methanol, 19.5 % distilled water, and 1 % formic acid) in 50 mg of the sample. Samples were mixed for 10 min and centrifuged at 9000 × *g* for 10 min. The extraction process was repeated two more times and the supernatants from the three extractions were pooled before the spectrophotometric analysis. The BP were extracted from the residue obtained after the extraction of the FP by alkaline hydrolysis with NaOH 2 M. The samples were kept for 12 h at room temperature to complete the hydrolysis. Then, the samples were mixed for 10 min and centrifuged at 9000 × *g* for 10 min; the supernatant was transferred to clean tubes and acidified with HCl 37 % (w/w) to reach pH 2. After centrifuging, 1 mL of supernatant was mixed with a solution of 99.9 % methanol and 0.1 % formic acid, and centrifuged (10 min at 9000 × *g*). The extraction process was repeated one more time. The supernatants from the two extractions were pooled and directly analyzed by spectrophotometric method. Both FP and BP determinations were carried out according to the Folin-Ciocalteu method adapted to a microplate format (Bobo-García et al., 2015). The absorbance was read at 760 nm using a Multiscan GO spectrophotometer (Thermo Scientific, Vantaa, Finland). Results were expressed as mg of gallic acid equivalents/g of whole grain flour.

The Abdel-Aal and Hucl (1999) method was used for the analysis of total AN compounds. Briefly, a 1.6 mL of solution of 85 % ethanol and 15 % hydrochloric acid 1 M was added to 100 mg of flour sample. Then, samples were mixed for 15 min and centrifuged at 27,200 × *g* for 30 min. Absorbance readings at 535 nm were taken using a Multiscan GO spectrophotometer (Thermo Scientific, Vantaa, Finland). The AN were quantified with a standard curve obtained for the cyanidin-3 glucoside. The results were expressed as μg of cyanidin-3 glucoside equivalents/g of whole grain flour.

2.3. NIRS analysis and calibration procedure

Two NIR spectrometers were used in this study. A NIRSystems 5000 scanning monochromator (FOSS, Hillerød, Denmark) with spectral range 1100 2500 nm using a PbS detector, located at the Universitat Autònoma de Barcelona (Bellatera, Spain); and a Vis-NIR spectrometer DS2500 (FOSS, Hillerød, Denmark) equipped with a monochromator with spectral range 400 2500 nm and a dual Si and PbS detector, located at the food laboratory of the Department of Agronomy, Food, Natural resources, Animals and Environment of the University of Padova (Legnaro, Italy). In both spectrometers the spectral data were collected in a diffuse reflection mode. The first one used a closed ring cup cell (35 mm diameter) with quartz glass windows containing 2-3 g of the sample and absorbance was recorded as log (1/reflectance) (log (1/R)) every 2 nm from 1108 to 2492 nm which gave 692 data points for each sample. Each sample was scanned twice, by manually rotating the sample cup approximately 180° relative to the previous scan. In order to obtain better accuracy in calibration, we used the mean of the two scans of each sample (average spectral data). The DS2500 used an open ring cup cell (40 mm diameter intern; FOSS cup) containing 3-4 g of the sample and absorbance was recorded as log (1/R) every 0.5 nm from 408 to 2492 nm in which 4138 data points were given for each sample. Each spectrum was the average of 32 sub-spectra collected during the automatic rotation of the FOSS cup.

Chemometric analysis was performed using WinISI 4.10 software (Infrasoft International, Port Matilda, PA, USA). Prior to calibration, log (1/R) spectra were corrected for the effects of scatter using the standard normal variate (SNV) and detrend (D) algorithms and by multiplicative scatter correction (MSC) to reduce the effects of the particle size. The prediction models were performed by the modified partial least squares regression (MPLS) and several combinations of scattering correction (NONE, D, SNV, SNV + D, MSC) and derivative (0,0,1,1; 1,4,4,1; 1,5,5,1;

1,8,8,1; 1,10,10,1; 2, 4,4,1; 2,5,5,1; 2,8,8,1; and 2,10,10,1; where the first digit is the number of the derivative, the second is the gap over which the derivative is calculated, the third is the number of data points in the first smoothing, and the fourth is the number of data points in the second smoothing) were tested.

One hundred and fifty samples were used for calibration (approximately 80 % of the total samples) and 36 random samples (around 20 % of the total samples) were separated previously and used as an external source of samples for validation. Different MPLS regressions were developed using different spectral range and spectral step: 1) NIRSystems 5000, range: 1108-2492 nm, 2 nm steps, 692 data points; 2) DS2500, range: 408-2492 nm, 0.5 nm steps, 4138 data points; 3) DS2500, range: 408-1092 nm, 0.5 nm steps, 1369 data points; and 4) DS2500, range: 1108-2492 nm, 0.5 nm steps, 2769 data points. In all scenarios, the same samples were used as a validation set. In the calibration set, chemical (t) or spectral (H) outliers (about 8% of the samples) were removed. The t outliers are samples that have a relationship between their reference values and spectra that is different from the relationship of the other samples in the set and with large residuals (t values > 2.2). The optimum model was selected by the following statistics: minimum standard error of calibration (SEC), minimum standard error of prediction (SEP), greatest coefficient of determination for calibration (R^{2CAL}), greatest coefficient of determination for validation (R^{2VAL}), the ratio of performance to deviation near 3.0 (RPD, defined as the ratio of standard deviation for the validation samples to the value of SEP), and the range error ratio near 10 (RER, defined as the ratio of the range in the reference data from the validation set to the SEP) (Williams, 2014; Williams and Sobering, 1996).

Moreover, in the validation set, reference values of each model were linearly regressed on the respective predicted value to obtain the linear regression coefficient (slope), to test if the slope differed from one, and to obtain the residuals using PROC REG of SAS ver. 9.4 (SAS Institute Inc., Cary, NC, USA). Then, the normality of the residuals was assessed and the average of the residuals (bias) was tested using PROC UNIVARIATE of SAS ver. 9.4 (SAS Institute Inc., Cary, NC, USA) to see if differed from zero.

3. Results & discussion

Table 1 shows the compositional data of the ground barley samples employed for the development of the calibration and validation equations in this study. Barley samples were selected to include the greatest possible genetic variability and were obtained in three different years to provide a broad range of bioactive components, which are considered basic principles to obtain good calibration models (De Marchi et al., 2018; Næs et al., 2002). Therefore, the distribution of samples obtained across the entire range (Table 1) is a good starting point for a robust calibration. All parameters were well represented in both calibration and validation matrices covering similar ranges and a relatively broad range as is recommended (Næs et al., 2002).

Average absorbance spectra for ground barley samples, captured by the two instruments (NIRSystems 5000 and DS2500), with the most relevant absorption bands indicated are shown in Fig. 1. In the visible region of the spectrum (400 700 nm), absorption was probably affected by the pericarp color of the barley samples (Dykes et al., 2014) as it suggests the observed peak at 667 nm (red light). In the NIRS region of the spectra, a first and defined peak was observed at 1200 nm and a wide peak at 1460 nm. Further peaks were found close to 1720 and 1770 nm. Moreover, there was a narrow peak at 1930 nm, a wide peak at 2100 and three more peaks at 2280, 2320 and 2490 nm. The average NIRS raw spectrum obtained followed a similar shape to those previously reported in whole kernel barley (Sohn et al., 2008) and ground barley samples (Dykes et al., 2014; Shi et al., 2019). According to previous reports, the characteristic bands of BG constituents can be detected in the regions from 1194 nm to 1290 nm and from 2260 nm to 2380 nm (Seefeldt et al., 2009), and phenolics and flavonoids should be detected in the regions 1415 1512 nm, 1650 1750 nm, and 1955 2035 nm (Han et al., 2017; Verardo et al., 2015; Zhang et al., 2008).

In the present study, calibrations by MPLS regression were performed for barley flour using the average spectral per sample and two NIR spectrometers with different spectral range and wavelength accuracy (Table 2). Different pre-treatments of spectral data were tested for their ability to remove or reduce disturbing effects not related to the chemical absorption of light. The model for each component shown in Table 2 is the best result from 45 models developed resulting from the evaluation of different pre-treatments (five scatter correction techniques and nine math treatments). The optimal spectral pre-treatments were the second derivative (2,4,4,1 and 2,5,5,1) of log (1/R) -except for BG

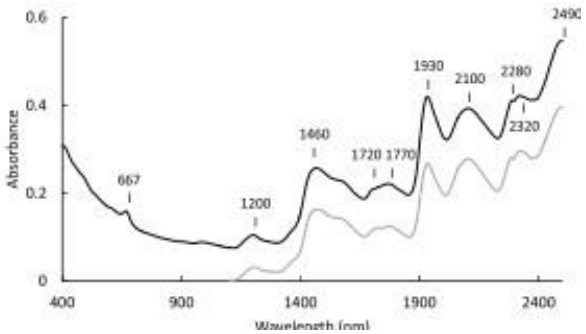


Fig. 1. Spectra of ground barley. In black, average vis/NIR spectrum using the DS2500; in grey, average NIR spectrum using the NIRSysems 5000.

Table 1
Flour barley population statistics of calibration and validation data set used.

Parameter	Calibration set				Validation set			
(Units)	N	Range	Mean	SD	n	Range	Mean	SD
β-glucan (g/100 g)	142	1.18 9.95	5.90	1.81	36	3.46 9.17	6.06	1.55
Arabinoxylan (g/100 g)	145	1.76 7.93	4.71	1.32	35	2.10 7.02	4.74	1.20
Bound Phenol (mg/g)[a]	150	1.70 6.76	3.65	1.05	36	1.91 6.37	3.76	1.18
Free Phenol (mg/g)[a]	150	1.38 4.15	2.41	0.58	36	1.38 3.51	2.35	0.52
Anthocyanin (μg/g)[b]	139	2.76 66.47	19.45	14.56	32	3.43 54.03	17.68	14.07

N = number of samples for calibration; n = number of samples for external validation; Range = interval between the minimum and maximum value of data set; SD = standard deviation.

[a] Phenolic content was expressed as mg of gallic acid equivalents/g.

[b] Anthocyanins compounds were expressed as μg of cyanidin-3 glucoside equivalents/g.

Table 2
Calibration and cross-validation fitting statistics for flour barley bioactive compounds prediction models using NIRSytems 5000 and DS2500 instruments.

Trait	[a]Math treatment	[b]Scatter correction	R^{2CAL}	SEC	R^{2CV}	SECV	SD/SECV
NIRSytems 5000 (1108 2492 nm at 2 nm intervals; 692 data points)							
β -glucan	1,4,4,1	SNV + D	0.966	0.346	0.941	0.435	4.16
Arabinoxylan	2,4,4,1	MSC	0.665	0.744	0.581	0.816	1.62
Bound Phenol	2,4,4,1	SNV + D	0.898	0.340	0.830	0.425	2.47
Free Phenol	2,4,4,1	SNV + D	0.672	0.319	0.515	0.377	1.54
Anthocyanin	2,5,5,1	MSC	0.839	5.807	0.614	8.578	1.70
DS2500 (408 2492 nm at 0.5 nm intervals; 4138 data points)							
β -glucan	1,4,4,1	SNV + D	0.949	0.402	0.921	0.488	3.71
Arabinoxylan	2,4,4,1	SNV + D	0.674	0.721	0.590	0.794	1.66
Bound Phenol	2,4,4,1	SNV + D	0.855	0.386	0.785	0.458	2.29
Free Phenol	2,4,4,1	SNV + D	0.704	0.301	0.615	0.335	1.73
Anthocyanin	2,5,5,1	MSC	0.660	6.920	0.564	8.174	1.78
DS2500 (408 1092 nm at 0.5 nm intervals; 1369 data points)							
β -glucan	1,4,4,1	SNV + D	0.825	0.724	0.751	0.850	2.13
Arabinoxylan	2,4,4,1	SNV + D	0.673	0.712	0.552	0.841	1.57
Bound Phenol	2,4,4,1	MSC	0.844	0.402	0.785	0.460	2.28
Free Phenol	2,4,4,1	MSC	0.633	0.300	0.401	0.375	1.55
Anthocyanin	2,5,5,1	MSC	0.856	5.309	0.616	8.292	1.76
DS2500 (1108 2492 nm at 0.5 nm intervals; 2769 data points)							
β -glucan	1,4,4,1	SNV + D	0.967	0.332	0.944	0.415	4.36
Arabinoxylan	2,4,4,1	SNV + D	0.563	0.811	0.502	0.856	1.54
Bound Phenol	2,4,4,1	SNV + D	0.807	0.438	0.765	0.484	2.17
Free Phenol	2,4,4,1	SNV + D	0.682	0.304	0.589	0.347	1.67
Anthocyanin	2,5,5,1	MSC	0.689	7.231	0.491	9.002	1.62

R^{2CAL} coefficient of determination for calibration; SEC standard error of calibration; R^{2CV} coefficient of determination for cross validation; SECV standard error of cross validation.

[a] Math treatment: derivative order, subtraction gap, first smoothing, second smoothing. [b] SNV standard normal variate, D detrend, MSC multiple scatter correction.

content which was the first derivative (1,4,4,1)- combined with SNV-D or MSC. These mathematical treatments are commonly used in food and cereal analysis by NIRS (Stubbs et al., 2010; Woodcock et al., 2008). The linear regression of measured versus predicted values for the best calibration equation for each bioactive compound (BG, AX, BP, FP, and AN) are represented in Fig. 2. After applying those calibration models to the validation dataset, slope of measure *vs* predict values did not differ from the unity, except the one selected for AN using DS2500, range 408-2492 nm at 0.5 intervals; residuals were normally distributed and bias did not differ from zero. The best predictive models built among all the components analyzed were obtained for BG and BP content, showing an R^{2CAL} of 0.97 and 0.90, respectively. Predictions are considered excellent when coefficient of determination is greater than 0.91, good when ranges from 0.82 to 0.90, approximate when is between 0.66 and 0.81, and poor when is less than 0.66 (Karoui et al., 2006). The worse predictive models were observed for AX and FP with $R^{2CAL} \leq 0.70$. As reported by Manley (2014), these apparently poor results could be related to the accuracy of the reference method and/or the narrow range of values encountered (Tables 1 and 2). The error of laboratory increases when more steps are involved and lower is the concentration of the trait to be predicted, which directly influences the accuracy of the prediction models (De Marchi et al., 2018).

We obtained excellent calibration models for BG content with both devices (instruments) and wavelength range (R^{2CAL}, 0.95 to 0.97; SEC, 0.33 to 0.40; SD/SECV, 3.7-4.4; Table 2). Only a slightly lower accuracy of the models for BG content was observed with the wavelength range from 408 to 1092 nm. That specific range is the one that considered the shorter wavelength region of the instrument Vis-NIR. The calibration model was still considered good (R^{2CAL}, 0.83), but it presented greater SEC (0.72) and lower SD/SECV (2.1). These results were in agreement with those reported by Schmidt et al. (2009) using a FT-NIR (R^{2CAL} = 0.96 0.98) and by Seefeldt et al. (2009) in barley flour samples with a dispersive NIRS in the region 1194 -1240 nm (R^{2CAL} = 0.94). Ringsted et al. (2017) showed the effect of BG range on the model performance to predict BG content in barley. Models with narrower BG range showed lower R^{2CAL} values than models with wider BG range -like in our case-, which achieved greater R^{2CAL} values (Ringsted et al., 2017). Some studies demonstrated the potential to discriminate barley flour or barley slice samples using long-wavelength (from 2200 to 2500 nm) based on their BG content (Ringsted et al., 2017; Seefeldt et al., 2009) which supports the slightly better results we obtained using the NIR region (1108-2492 nm) than the vis-NIR region (408-1092 or 408 2498 nm).

Validation statistics (Table 3) for the best-fitting equations previously selected for BG (Table 2) showed a great accuracy of the models using both devices and different wavelength range (RPD, 3.2-3.9; RER, 11.8-14.5; SEP, 0.39-0.49). However, validation statistics considering only from 408 to 1092 nm were poorer (RPD, 2.1; RER 7.6; SEP, 0.75). Based on Williams (2014), for complex feed matrix, a prediction model with an RPD ≤ 1.9 is considered to be not suitable; values between 2.0 and 2.4 are considered poor and only adequate for rough screening purposes, values between 2.5 and 2.9 could be applied for screening, and values ≥ 3.0 (or RER > 10) indicate good prediction and can be used for quantitative analysis. Those results confirm the high precision of the BG prediction model developed using both devices and a wavelength above 1092 nm since they provided higher values than the minimum recommended for prediction uses. In contrast, Sohn et al. (2007) reported a much lower R^{2CAL} (0.67) and RPD (1.6) for BG using a dispersive NIR spectroscopy (NIR region, 1100-2498 nm) than we did, but the study was focused on barley for bioethanol production, thus, barley samples rich in BG content were not included. A better prediction model adequate for quality control (RPD = 3.5) was reported by Blakeney and Flinn (2005) using a sample set of grains and pulses, while a RPD of 4-5.6 was reported in naked barley by Schmidt et al. (2009) using FT-NIR. Although, a lower RPD (2.1-2.3) was achieved using dispersive NIR (1100-2500 nm) (Schmidt et al., 2009).

Calibrations developed for AX content were unsatisfactory ($R^{2CAL} \leq$ 0.67; Table 2) considering the studied instruments and wavelength ranges. In the same way, validation statistics revealed that prediction models were not suitable for prediction purposes (RPD ≤ 1.5; RER ≤ 6.0; SEP ≤ 0.9; Table 3). A better accuracy for AX content (R^{2CV} = 0.84; RPD = 2.5) was reported previously on a set of samples of cereals grains and pulses including a wider diversity (i.e., with diverse genetic and

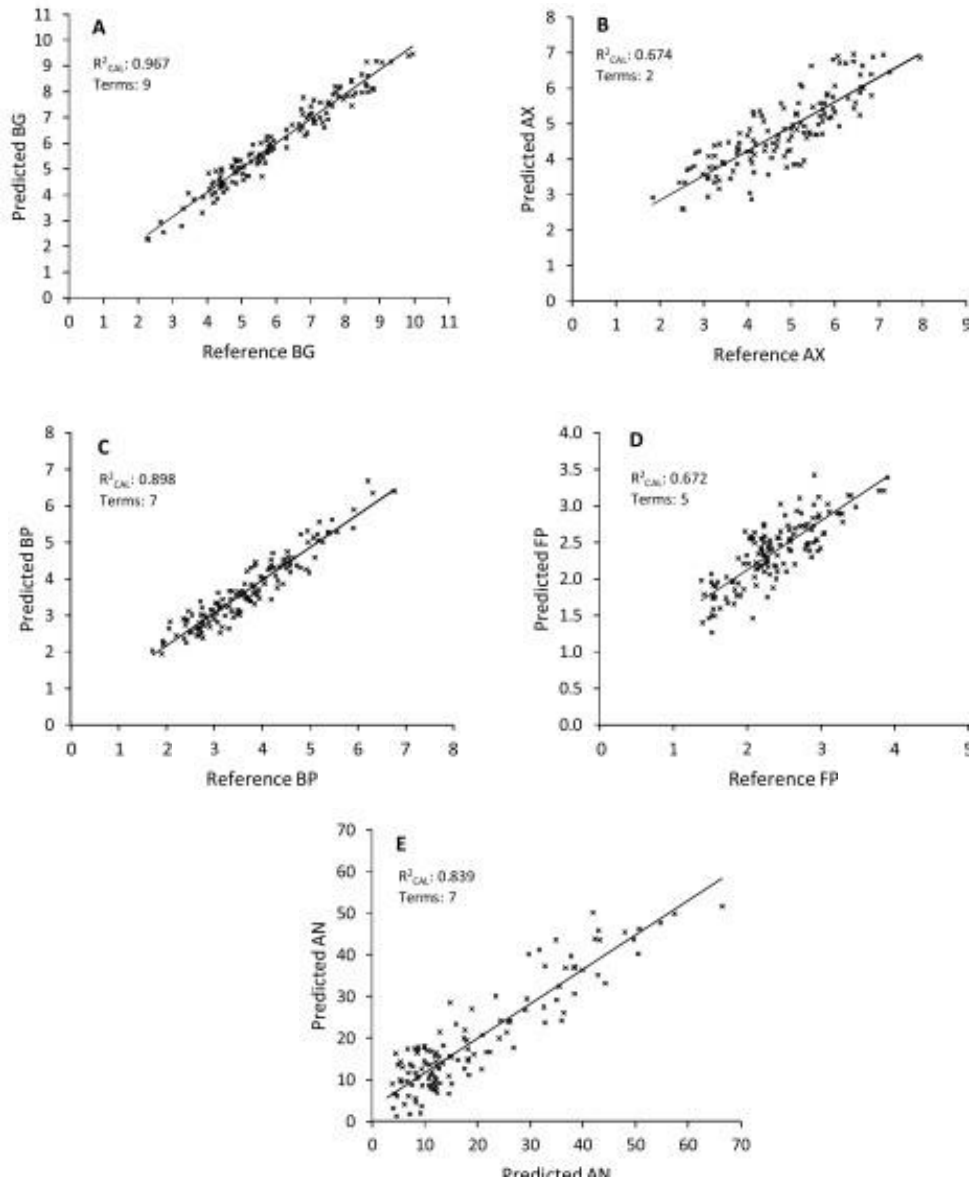


Fig. 2. Linear regression plot of measured versus predicted values of the calibration dataset for: (A) β-glucan (BG), g/100 g; (B) Arabinoxylan (AX), g/100 g; (C) Bound phenol (BP), mg of gallic acid equivalents/g; (D) Free phenol (FP), mg of gallic acid equivalents/g; and (E) Anthocyanin (AN), μg of cyanidin-3 glucoside equivalents/g. R^{2CAL}: coefficient of determination in calibration.

environmental backgrounds) (Blakeney and Flinn, 2005). Although NIR spectroscopy was capable of detecting AX content in plant materials (Blakeney and Flinn, 2005; Kačuráková et al., 1994), to the best of our knowledge, no study has obtained satisfactory prediction models for AX in barley flour.

In the current study, the best calibration equation for phenolic acids was obtained for BP using the 1108 2492 nm spectral range and 2 nm steps (R^{2CAL} = 0.90; SD/SECV = 2.5; Table 2). The use of the instrument with more resolution (0.5 nm steps) and that included the visible region (408-2492 nm spectral range) slightly decreased the R^{2CAL} and the SD/SECV (0.86 and 2.3, respectively; Table 2). According to previous reports, the characteristic bands of phenolics and flavonoids should be detected in wavelength regions from 1415 nm to 1512 nm, 1650-1750 nm, and from 1955 to 2035 nm (Cozzolino et al., 2004; Verardo et al., 2015; Zhang et al., 2008), thus, the inclusion of the visible wavelength seems to impair the prediction model. The R^{2VAL} and the RPD index for BP content using the 1108-2492 nm spectral range and 2 nm were 0.9 and 3.2, respectively, which indicated the effectiveness of the method. However, the accuracy of the prediction models in external validation was lower for FP (R^{2VAL} = 0.6; RPD = 1.7) and AN (R^{2VAL} = 0.7; RPD = 2.1) content, which suggested a greater variation between the reference values and NIR predictions for FP and for AN than for BP content. Sometimes, low coefficient of determinations can be observed in samples with low or non-detectable concentrations of the component and/or narrow range, thus making more difficult to quantify and to predict them. Anthocyanins belong to the flavonoids group and provide the purple, blue, and red shades in many plants, including barley seeds. Despite the relationship between anthocyanins and color, the predictive models obtained did not improve ostensibly when the visible range was included.

Phenolic compounds in cereal grains are either in free or bound forms. Generally, free phenolic compounds are proanthocyanidins or flavonoids, whereas bound phenolic compounds are ester-linked to cell wall polymers and mainly consist of ferulic acid and its oxidatively coupled dimers (Bonoli et al., 2004). In ground rice samples, Zhang et al. (2008) determined total phenolic and flavonoid content achieving R^{2VAL} values of 0.86 and 0.38 and RPD values of 2.6 and 1.3, respectively, using a dispersive NIRS. In whole sorghum grain, Dykes et al. (2014)

Table 3
Validation fitting statistics bioactive for flour barley bioactive compounds prediction models using NIRSytems 5000 and DS2500 instruments.

Trait	R^{2VAL}	SEP	Bias	Slope	RPD	RER
NIRSytems 5000 (1108 2492 nm) 2 nm						
β -glucan	0.912	0.486	0.049	0.918	3.19	11.75
Arabinoxylan	0.568	0.834	0.246	0.805	1.44	5.90
Bound Phenol	0.893	0.371	0.053	0.983	3.18	12.02
Free Phenol	0.632	0.307	0.025	1.239	1.69	6.94
Anthocyanin	0.675	6.707	0.899	0.808	2.10	7.54
DS2500 (408 2492 nm) 0.5 nm						
β -glucan	0.920	0.435	0.048	0.963	3.56	13.13
Arabinoxylan	0.578	0.868	0.325	0.821	1.38	5.67
Bound Phenol	0.842	0.449	0.046	0.988	2.63	9.93
Free Phenol	0.635	0.327	0.031	0.821	1.59	6.51
Anthocyanin	0.541	8.688	0.220	0.682	1.62	5.82
DS2500 (408 1092 nm) 0.5 nm						
β -glucan	0.766	0.754	0.023	0.962	2.06	7.57
Arabinoxylan	0.545	0.833	0.248	0.871	1.44	5.91
Bound Phenol	0.764	0.552	0.017	0.922	2.14	8.08
Free Phenol	0.624	0.328	0.091	0.935	1.59	6.49
Anthocyanin	0.630	8.837	0.361	0.887	1.59	5.73
DS2500 (1108 2492 nm) 0.5 nm						
β -glucan	0.937	0.394	0.013	1.014	3.93	14.49
Arabinoxylan	0.459	0.825	0.112	0.739	1.45	5.96
Bound Phenol	0.830	0.471	0.004	1.092	2.51	9.47
Free Phenol	0.637	0.324	0.046	0.849	1.60	6.57
Anthocyanin	0.471	9.644	2.210	0.834	1.46	5.25

R^{2VAL} coefficient of determination for external validation; SEP standard error of prediction; RPD ratio of performance to deviation (SD/SEP); RER range error ratio (Range/SEP).

obtained values of R^{2VAL} of 0.86 and 0.68 for total phenols and for 3-deoxyanthocyanidins, respectively, using a vis/NIR spectrometer. In grain ground barley samples, Verardo et al. (2015) reported R^{2VAL} values of 0.96 to determine proanthocyanidin using the spectral range (1100 2500 nm) and FT-NIR spectroscopy, and more recently, Han et al. (2017) obtained R^{2VAL} and RPD values for total phenolics content of 0.95 and 3.2, respectively, and for free *p*-coumaric acid content of 0.96 and 3.6, respectively, using a dispersive NIR instrument; values slightly higher to the ones found in the present study. To the best of our knowledge, there are no references available on barley grain to compare the accuracy of our prediction equations for BP, FP, and AN.

4. Conclusion

The study investigated the feasibility of using two different NIRS instruments, resolution, and wavelength range, combined with chemometrics for the determination of non-starch polysaccharides, phenols compounds, and anthocyanins in a wide range of barley flour. Different spectral pretreatment techniques have been tried to construct acceptable models. It was concluded that the NIRS models developed were sufficiently accurate to predict BG and BP contents in barley grain, poor to predict AN, and not reliable for the prediction of FP and AX content. Our results highlighted that both spectral range and spectral steps influence the accuracy of the models, obtaining the best results retaining only the 1108-2492 nm region and using the 0.5 nm steps for BG and 2 nm steps for the rest of components. Despite the relationship between AN and color, the predictive models obtained apparently did not improve when the visible range was included. The AX prediction model was the component with worst results, probably due to the complexity of this fraction and/or the narrow range of our values.

Author statement

All co-authors have approved the manuscript and there is no financial interest to report. We confirm that this manuscript has not been published elsewhere and is not under consideration by another journal.

CRediT authorship contribution statement

Elena Albanell: Conceptualization, Methodology, Writing - original draft, Writing - review & editing, Funding acquisition. Mariona Martínez: Methodology, Formal analysis, Writing - review & editing. Massimo De Marchi: Supervision, Funding acquisition, Writing - review & editing. Carmen L. Manuelian: Conceptualization, Data curation, Formal analysis, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This research was funded by MINECO/FEDER (AGL2015-69435-C3-3-R) project (Spain). Vis-NIR spectrometer DS2500 was funded by Progetto di Eccellenza "Centro per l'Agricoltura, la Sostenibilità e gli Alimenti" (CASA), CUP C26C18000190001, MIUR (Italy).

References

Abdel-Aal, E.S.M., Hucl, P., 1999. A rapid method for quantifying total anthocyanins in blue aleurone and purple pericarp wheats. Cereal Chem. 76 (3), 350-354. https://doi.org/10.1094/CCHEM.1999.76.3.350.

Alfieri, M., Cabassi, G., Habyarimana, E., Quaranta, F., Balconi, C., Redaelli, R., 2019. Discrimination and prediction of polyphenolic compounds and total antioxidant capacity in sorghum grains. J. Near Infrared Spectrosc. 27 (1), 46-53. https://doi.org/10.1177/0967033518825351.

Bartłomiej, S., Justyna, R.K., Ewa, N., 2012. Bioactive compounds in cereal grains occurrence, structure, technological significance and nutritional benefits – a review. Food Sci. Technol. Int. 18 (6), 559-568. https://doi.org/10.1177/1082013211433079.

Blakeney, A.B., Flinn, P.C., 2005. Determination of non-starch polysaccharides in cereal grains with near-infared reflectance spectroscopy. Mol. Nutr. Food Res. 49 (6), 546-550. https://doi.org/10.1002/mnfr.200500038.

Bobo-García, G., Davidov-Pardo, G., Arroqui, C., Vírseda, P., Marín-Arroyo, M.R., Navarro, M., 2015. Intra-laboratory validation of microplate methods for total phenolic content and antioxidant activity on polyphenolic extracts, and comparison with conventional spectrophotometric methods. J. Sci. Food Agric. 95 (1), 204-209. https://doi.org/10.1002/jsfa.6706.

Bonoli, M., Verardo, V., Marconi, E., Caboni, M.F., 2004. Antioxidant phenols in barley (*Hordeum vulgare* L.) flour: comparative spectrophotometric study among extraction methods of free and bound phenolic compounds. J. Agric. Food Chem. 52 (16), 5195-5200. https://doi.org/10.1021/jf040075c.

Cozzolino, D., Kwiatkowski, M.J., Parker, M., Cynkar, W.U., Dambergs, R.G., Gishen, M., Herderich, M.J., 2004. Prediction of phenolic compounds in red wine fermentations by visible and near infrared spectroscopy. Anal. Chim. Acta 513 (1), 73-80. https://doi.org/10.1016/j.aca.2003.08.066.

De Marchi, M., Penasa, M., Zidi, A., Manuelian, C.L., 2018. Invited review: use of infrared technologies for the assessment of dairy products—applications and perspectives. J. Dairy Sci. 101 (12), 10589-10604. https://doi.org/10.3168/jds.2018-15202.

De Sá, R.M., Palmer, G.H., 2006. Analysis of β-glucan in single grains of barley and malt using NIR-spectroscopy. J. Inst. Brew. 112 (1), 9-16. https://doi.org/10.1002/j.2050-0416.2006.tb00701.x.

Dykes, L., Hoffmann, L., Portillo-Rodriguez, O., Rooney, W.L., Rooney, L.W., 2014. Prediction of total phenols, condensed tannins, and 3-deoxyanthocyanidins in sorghum grain using near-infrared (NIR) spectroscopy. J. Cereal Sci. 60 (1), 138-142. https://doi.org/10.1016/j.jcs.2014.02.002.

Gordon, R., Chapman, J., Power, A., Chandra, S., Roberts, J., Cozzolino, D., 2019. Mid-infrared spectroscopy coupled with chemometrics to identify spectral variability in Australian barley samples from different production regions. J. Cereal Sci. 85 (May 2018), 41-47. https://doi.org/10.1016/j.jcs.2018.11.004.

Gupta, M., Abu-Ghannam, N., Gallaghar, E., 2010. Barley for brewing: characteristic changes during malting, brewing and applications of its by-products. Compr. Rev. Food Sci. Food Saf. 9 (3), 318-328. https://doi.org/10.1111/j.1541-4337.2010.00112.x.

Han, Z., Cai, S., Zhang, X., Qian, Q., Huang, Y., Dai, F., Zhang, G., 2017. Development of predictive models for total phenolics and free p-coumaric acid contents in barley grain by near-infrared spectroscopy. Food Chem. 227, 342-348. https://doi.org/10.1016/j.foodchem.2017.01.063.

Kačuráková, M., Ebringerová, A., Hirsch, J., Hromádková, Z., 1994. Infrared study of arabinoxylans. J. Sci. Food Agric. 66 (3), 423-427. https://doi.org/10.1002/jsfa.2740660323.

Karoui, R., Mouazen, A.M., Dufour, É., Pillonel, L., Picque, D., Bosset, J.-O., De Baerdemaeker, J., 2006. Mid-infrared spectrometry: a tool for the determination of

chemical parameters in Emmental cheeses produced during winter. Lait 86, 83-97. https://doi.org/10.1051/lait:2005040.
Li, J., Kaneko, T., Qin, L.Q., Wang, J., Wang, Y., 2003. Effects of barley intake on glucose tolerance, lipid metabolism, and bowel function in women. Nutrition 19 (11-12), 926-929. https://doi.org/10.1016/S0899-9007(03)00182-5.
Næs, T., Isaksson, T., Fearn, T., Davies, T., 2002. A User-Friendly Guide to Multivariate Calibration and Classification. NIR Publications (ed.), Chichester.
Osborne, B.G., 2000. Near-infrared spectroscopy in food analysis. Encyclopedia of Analytical Chemistry, pp. 1-14. https://doi.org/10.1002/9780470027318.a1018.
Ringsted, T., Ramsay, J., Jespersen, B.M., Keiding, S.R., Engelsen, S.B., 2017. Long wavelength near-infrared transmission spectroscopy of barley seeds using a supercontinuum laser: prediction of mixed-linkage beta-glucan content. Anal. Chim. Acta 986, 101-108. https://doi.org/10.1016/j.aca.2017.07.008.
Schmidt, J., Gergely, S., Schönlechner, R., Grausgruber, H., Tömösközi, S., Salgó, A. Berghofer, E., 2009. Comparison of different types of NIR instruments in ability to measure β-glucan content in naked barley. Cereal Chem. 86 (4), 398-404. https://doi.org/10.1094/CCHEM-86-4-0398.
Seefeldt, H.F., Blennow, A., Jespersen, B.M., Wollenweber, B., Engelsen, S.B., 2009. Accumulation of mixed linkage (1 → 3) (1 → 4)-β-d-glucan during grain filling in barley: a vibrational spectroscopy study. J. Cereal Sci. 49 (1), 24-31. https://doi.org/10.1016/j.jcs.2008.06.012.
Shi, H., Schwab, W., Liu, N., Yu, P., 2019. Major ergot alkaloids in naturally contaminated cool-season barley grain grown under a cold climate condition in western Canada, explored with near-infrared (NIR) and fourier transform mid-infrared (ATR-FT/MIR) spectroscopy. Food Control 102 (January), 221-230. https://doi.org/10.1016/j.foodcont.2019.03.025.
Sohn, M., Himmelsbach, D.S., Barton, F.E., Griffey, C.A., Brooks, W., Hicks, K.B., 2007. Near-infrared analysis of ground barley for use as a feedstock for fuel ethanol production. Appl. Spectrosc. 61 (11), 1178-1183. https://doi.org/10.1366/000370207782597148.
Sohn, M., Himmelsbach, D.S., Barton, F.E., Griffey, C.A., Brooks, W., Hicks, K.B., 2008. Near-infrared analysis of whole kernel barley: comparison of three spectrometers. Appl. Spectrosc. 62 (4), 427-432. https://doi.org/10.1366/000370208784046768.
Stubbs, T.L., Kennedy, A.C., Fortuna, A.M., 2010. Using NIRS to predict fiber and nutrient content of Dryland cereal cultivars. J. Agric. Food Chem. 58 (1), 398-403. https://doi.org/10.1021/jf9025844.
Verardo, V., Cevoli, C., Pasini, F., Gómez-Caravaca, A.M., Marconi, E., Fabbri, A., Caboni, M.F., 2015. Analysis of oligomer proanthocyanidins in different barley genotypes using high-performance liquid chromatography-fluorescence detection-mass spectrometry and near-infrared methodologies. J. Agric. Food Chem. 63 (16), 4130-4137. https://doi.org/10.1021/acs.jafc.5b01425.
Williams, P., 2014. Tutorial: the RPD statistic: a tutorial note. NIR News 25 (1), 22. https://doi.org/10.1255/nirn.1419.
Williams, P.C., Sobering, D.C., 1996. How do we do it: a brief summary of the methods we use in developing near infrared calibrations. Near Infrared Spectroscopy: The Future Waves, pp. 185-188.
Woodcock, T., Downey, G., O'Donnell, C.P., 2008. Better quality food and beverages: the role of near infrared spectroscopy. J. Near Infrared Spectrosc. 16 (1), 1-29. https://doi.org/10.1255/jnirs.758.
Zhang, C., Shen, Y., Chen, J., Xiao, P., Bao, J., 2008. Nondestructive prediction of total phenolics, flavonoid contents, and antioxidant capacity of rice grain using near-infrared spectroscopy. J. Agric. Food Chem. 56 (18), 8268-8272. https://doi.org/10.1021/jf801830z.

Universitat de Lleida
Escola Tècnica Superior
d'Enginyeria Agrària