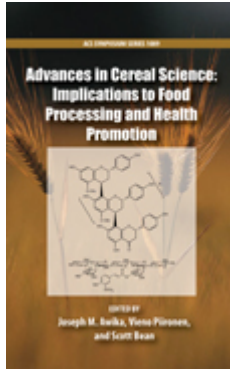
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Advances in Cereal Science: Implications to Food Processing and Health Promotion
EDITED BY
Joseph M. Awika, Vieno Piironen, and Scott Bean

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Foreword

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Before agreeing to publish a book, the proposed table of contents is reviewed for appropriate and comprehensive coverage and for interest to the audience. Some papers may be excluded to better focus the book; others may be added to provide comprehensiveness. When appropriate, overview or introductory chapters are added. Drafts of chapters are peer-reviewed prior to final acceptance or rejection, and manuscripts are prepared in camera-ready format.

As a rule, only original research papers and original review papers are included in the volumes. Verbatim reproductions of previous published papers are not accepted.

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Chapter 1

Major Cereal Grains Production and Use around the World

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Cereal grains have been the principal component of human diet for thousands of years and have played a major role in shaping human civilization. Around the world, rice, wheat, and maize, and to a lesser extent, sorghum and millets, are important staples critical to daily survival of billions of people. More than 50% of world daily caloric intake is derived directly from cereal grain consumption. Most of the grain used for human food is milled to remove the bran (pericarp) and germ, primarily to meet sensory expectations of consumers. The milling process strips the grains of important nutrients beneficial to health, including dietary fiber, phenolics, vitamins and minerals. Thus, even though ample evidence exists on the health benefits of whole grain consumption, challenges remain to developing food products that contain significant quantities of whole grain components and meet consumer expectations . This book presents some of the latest research endeavors that aim to improve our understanding of how the chemistry of various grain components can be manipulated to improve contribution of cereals to human health. Most of the topics are based on the Cereal Grains Symposium, at the 2011 American Chemical Society held in Anaheim, CA, March 27-31.

Introduction

Cereal grains have been a primary source of nourishment for humans for thousands of years. From domestication of rice about 10,000 years ago in Yangtze

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Valley, China (*1*), to domestication of maize (corn) in Southern Mexico/Central America and wheat in the Fertile Crescent of the Near East around the same time (*2*) cereal grains have contributed immensely to transforming human civilization. Today, cereal grains are the single most important source of calories to a majority of the world population. Developing countries depend more on cereal grains for their nutritional needs than the developed world. Close to 60% of calories in developing countries are derived directly from cereals, with values exceeding 80% in the poorest countries. By comparison, approximately 30% of calories in the developed world are derived directly from cereals (*3*). However, even in these more affluent societies that rely less on direct cereal consumption, cereals remain the most important food commodity, since they supply most of the nutrients for the livestock that form a major part of diet in these regions.

In regions like the U.S.A., cereals contribute significant additional indirect calories as sweeteners, particularly corn syrup-based sweeteners. For example, in the U.S.A., three times more corn is used for production of sweeteners and food starch, than is directly consumed by humans (including beverage alcohol). This often leads to underestimation of actual human per capita consumption, since most data only consider directly consumed portion. Increasingly, grains are also being used to produce industrial ethanol, most of which goes into fuels. For example, in the U.S.A., ethanol production (mostly from corn) increased 8-fold from 6.2 billion liters in 2000 to 50 billion liters in 2010 (*4*). This has somewhat stroked controversy owing to the rapid rise in grain commodity prices that has resulted from the increased demand of grains for bioethanol production. Other questions on economic feasibility and environmental impact of bioethanol production from grains remain. Developments in cellulosic ethanol production technology will go a long way in alleviating some of the concerns.

The type of grains produced around the world depends on various factors, the most important being environmental, cultural, and economic. Temperature and water availability are probably the most critical environmental factors that determine crops grown in a given region. In regions where water availability is non-limiting, rice, and to some extent maize, tend to dominate. Rice if probably the least water use efficient of all cereal crops, and is often produced under flooding conditions, thus is most susceptible to water deficiency. In most regions where rice is produced, large bodies of easily accessible fresh water or ample rainfall must be available. This typifies the regions of Asia where most of the world rice is produced.

Maize is relatively susceptible to drought as well, though not to the same extent as rice. Both maize and rice cannot withstand frost and must be grown in warm environments. Thus in the temperate regions, these crops are grown in spring and harvested in the summer. Wheat, on the other hand, is grown in a wider range of environments, from relatively limited water availability to high water availability. In addition, wheat can withstand a wide temperature range, and is thus widely produced in the temperate regions both in the winter and spring. Wheat is also commonly grown in warmer regions; however, higher temperatures during flowering and grain filling can significantly depress wheat yield. In regions where drought stress is a major issue (e.g., semi arid environments in Africa and India), sorghum and millet, two relatively drought tolerant crops, are usually commonly

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grown. Barley, the other major grain produced, is more tolerant of cold climates, and is thus mostly produced in northern Europe and northern parts of the U.S.A. and Canada.

Table 1. Major cereal grain world production statistics (2010). Sources: (*4–6*)

Commodity	*Production area (Million ha)*	*Production (MMT)*[a]	*International trade (MMT)*	*Percent grain entering international trade*
Maize	160	825	94	11.3
Wheat	226	650	122	18.8
Rice[b]	162	680 (440)	(30)	6.8
Barley	54	150	17.3	11.5
Sorghum	40	60	6.3	10.5

[a] MMT – Million metric tons [b] paddy rice (values in parentheses represent polished rice).

Major Cereal Production and Use

The three most important food crops in the world are rice, wheat, and maize (corn). The three cereal grains directly contribute more than half of all calories consumed by human beings. In addition, other minor grains like sorghum and millet are particularly major contributors of overall calorie intake in certain regions of the world, particularly semi-arid parts of Africa and India. For example, sorghum and millet contribute up to 85% of daily caloric intake in Burkina Faso and Niger (*5*). A large part of cereal grain production (particularly corn, barley, sorghum, and oats) also go into livestock feed, thus indirectly contributing to human nutrition. The overall importance of the major cereal grains to world food basket is summarized in the following sections.

Rice

Rice is the single most important source of calories for humans. Among cereal, rice is grown mainly for direct human consumption with very little making it to other uses. Rice contributes approximately 21% of world per capita caloric intake, and 27% of per capita calories in the developing countries. In highest consumption countries, Vietnam, Cambodia and Myanmar, up to 80% of caloric intake is derived from rice. Of the 440 million metric tons (MMT) of polished rice produced in the world in 2010 (Table 1), 85% went into direct human food supply (*5*). By contrast, 70% of wheat and only 15% of maize production was directly consumed by humans. Rice production (and consumption) is highly localized; Asia produces the vast majority 92% of world rice (*5*), with China and India accounting for 50% of world rice production in 2010 (Table 2). In some Asian

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countries, e.g., Cambodia, up to 90% of agricultural land is dedicated to rice production.

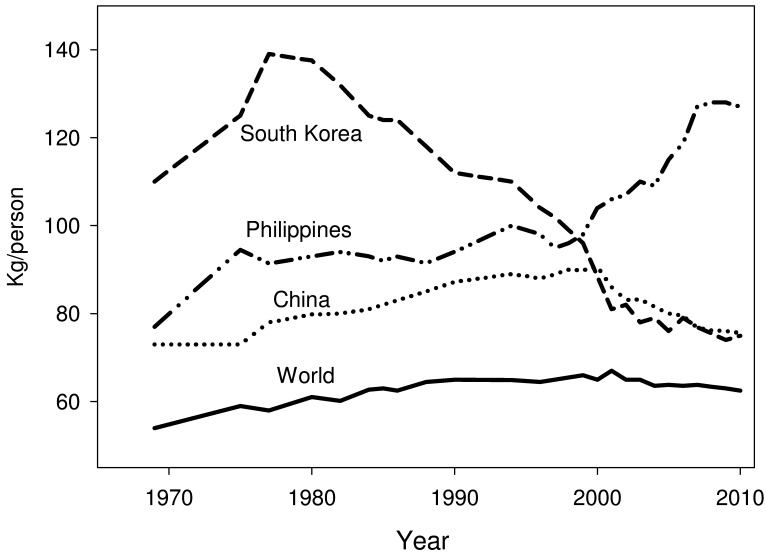

Figure 1. Trends in per capita rice consumption globally and in selected Asian countries (4, 5, 7).

The world average per capita consumption of rice in 2009 was 63 kg (milled), with the highest intake in Asian countries (90% of world rice production). Overall, rice consumption varies widely by region: For example average annual per capita consumption in high use countries, Bangladesh, Indonesia, Myanmar, Thailand, and Vietnam, was 150 – 226 kg in 2009. By contrast, low use countries like Argentina, most of Europe, and Mexico averaged 6 – 8 kg annual per capita consumption. Only a small fraction of world rice production (6.8%) enters international trade (Table 1), as most production is used to meet local demand. This indicates that most non-rice producing countries generally consume limited quantities of rice.

Because relatively little of the world rice production makes it into international trade, it is predicted that rice demand will decline as poor economies in Asia become more affluent and begin diversifying their diet. For example, per capita annual rice (polished) consumption in South Korea has declined steadily from a high of about 140 kg in late 1970s to 75 kg in 2010 (Figure 1). In China, per capita annual consumption has been on the decline since 2000; over the same period, world per capita consumption declined from 66 kg to 63 kg (Figure 1). However, future overall global per capita rice food use may not be that easy to predict; significant increase in rice consumption is being witnessed in some high use Asian countries, like the Philippines (Figure 1) and other world regions. Many countries in Sub Saharan Africa are experiencing significant growth in per capita rice consumption as their economies improve and they seek to diversify their diet

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from traditional maize and sorghum/millet. For example, in Ivory Coast, per capita rice consumption increased from 67 kg to 76 kg between 2000 and 2010 (*5*).

Table 2. Major world producers of cereal grains (2009/2010 data). Source: (*5*, *6*)

Country	*Production (MMT)*	*Production as a percent of world output*	*Average yield (Ton/ha)*
Maize			
U.S.A.	333	40.4	10.3
China	168	20.4	5.4
Brazil	51	6.2	3.7
Mexico	20	2.4	2.1
India	17	2.0	2.8
Wheat			
China	115	17.7	4.8
India	81	12.5	2.8
Russia	62	9.5	2.3
U.S.A.	60	9.2	3.0
France	38	5.8	7.5
Rice (milled)			
China	137	31.1	6.6
India	89	20.2	3.0
Indonesia	36	8.2	5.0
Bangladesh	31	7.0	3.9
Vietnam	25	5.7	5.2

Wheat

Wheat is a close second to rice as the most important source of calories for humans. Wheat is also the most important source of dietary protein for humans, though like other cereals, its protein is deficient in essential amino acids, especially lysine. In general, wheat is more adaptable to a wide range of growth conditions than other major cereal crops, and is thus the most widely cultivated food plant in the world with 226 million ha cultivated in 2009/2010, producing 650 MMT of grain (Table 1). China is the world largest producer, with China and India producing approximately 30% of world wheat in 2010 (Table 2). On a per capita basis, however, Australia is the largest wheat producer, at about 1.2 MMT per capita in 2009.

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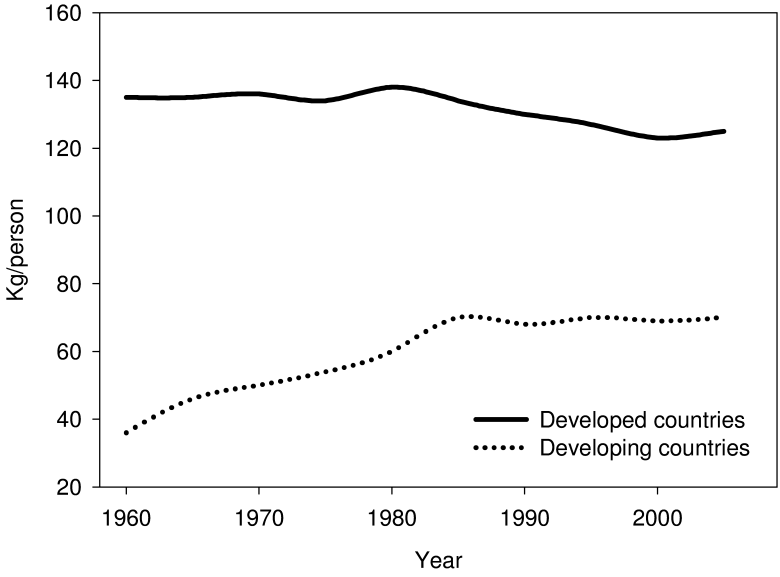

Figure 2. Trends in per capita food use of wheat in developing and developed countries. Based on data from (4, 8).

Like rice, per capita consumption of wheat flour varies widely by region; in countries like Egypt, Algeria, Israel, and many others in the Middle East and Eastern Europe, per capita consumption of wheat flour is high and often exceeds 150 kg per year (*5*). In other countries that rely more heavily on other cereals, per capita consumption is much lower (averaging 35 kg in Central America, and 17 kg in Sub Saharan Africa (*9*, *10*). Per capita average global food consumption of wheat was about 66 kg in 2010 (which translates to approximately 48 – 50 kg wheat flour) and is expected to remain constant over the next decade. Developing countries use more than 80% of their wheat supply (including imports) for food, compared to developed countries that use less than 50% (*3*).

Even though global per capita food use of wheat has held relatively steady over the past 50 years or so, consumption in developing countries has increased significantly during this time (Figure 2), offsetting the overall declining consumption in the developed world. For example, per capita consumption of wheat flour in several Asian and Sub Saharan Africa countries has been growing steadily; India consumed about 40 kg wheat per person per year in 1970, and almost 66 kg/person/year in 2007. In many Asian countries, increase in wheat consumption has been partly at the expense of rice. This is attributed to diversification of diets as a result of growing economies and increased global trade.

In the U.S.A., earlier trends of wheat flour consumption followed similar patterns typically observed in developing economies, with steady decline observed from 1890s to 1960s (Figure 3) as American economy steadily grew and people

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switched more and more to animal based products. However, the trend reversed beginning in the 1960s as health concerns of animal product consumption, particularly the cholesterol and heart disease link based on studies at the time, led to increasing consumption of wheat flour and other plant based products that lasted until the late 1990s when the trend reversed once more. The decline that began around the year 2000 was largely due to a low carbohydrate dietary fad, popularized by weight loss programs. These diet programs posited (with much controversy) that carbohydrates consumption was primarily responsible for increasing incidences of obesity and associated health problems in the U.S.A., and that severely limiting carbohydrate intake would reverse the trend. The low carbohydrate dietary trend faded rather quickly, and a major contributor to the failure was the limited sensory appeal of consistent intake of low carbohydrate products. If anything, the low carbohydrate fad confirmed that even though man cannot live by bread alone, man definitely cannot live without bread.

The U.S.A. experience with wheat consumption clearly indicates that health concerns are a major driver of consumer behavior in terms of their food choices. It also illustrates that potential health benefits alone are not enough to sustain a trend in the long term. Food must also meet consumer sensory needs and expectations; this has been a major bottleneck in attempts to provide healthier foods to consumers. The current efforts to position whole grain-based products at the forefront of healthy eating are likely to go nowhere if these lessons from the past are not taken to heart.

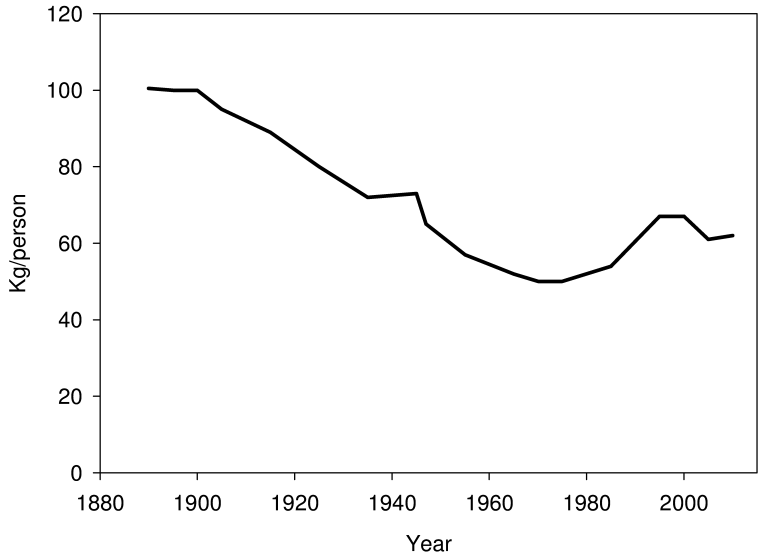

Figure 3. Per capita wheat flour consumption trend in the U.S.A. Based on USDA-ERS data (4).

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Maize (Corn)

In terms of production, maize is the leading crop in the world, with 825 MMT produced in 2010 (Table 1). The U.S.A. is by far the largest producer of maize, accounting for 40% of world production in 2010, followed by China at 20%. However, unlike the other two major cereals, maize is mostly used as animal feed, with only 15% of grain used for food. In Africa and Latin America, maize is a major staple, with more than 90% of the maize in Africa used for food and an average per capita consumption of about 50 kg. Africa consumes about 30% of world food maize, with Sub Saharan Africa consuming the vast majority (*5*). East and Southern Africa consume more maize per capita than the rest of Africa; for example, countries like Malawi, Lesotho, Zambia, and Kenya, consume 90 – 180 kg of maize per person annually. In countries like Mexico, maize is a particularly important staple, contributing approximately 45% of the daily caloric intake (mostly as tortillas), compared to about 10% for all other grains combined (*5*).

In the U.S.A., maize use for human food is fairly limited, with per capita consumption reported as 15 kg in 2009 (*4*), or about 2.1% of the more than 700 kg per capita production. However, this data only represents direct consumption as grits, breakfast cereal, snacks, etc. The single major food use of corn in the U.S.A., as sweetener, is, not included in the per capita data; corn sweetener use in the U.S.A. stood at 30 kg dry weight per capita in 2009, after peaking at about 37 kg in 2000 (*4*). True per capita food use of maize in the U.S.A. is thus close to 60 kg, making it an important food commodity. The use of corn as sweetener is generally categorized under 'industrial' uses.

A dramatic shift in maize utilization with important consequences to world food prices has occurred in the past 10 years or so. The U.S.A., which accounts for about 60% of world maize export, saw a dramatic increase in use of maize for fuel ethanol production. Between 2000 and 2010, use of maize for ethanol production increased 8-fold from 16 MMT to 128 MMT, partly necessitated by the high oil prices during this time, as well as government policy towards energy independence. Fuel ethanol currently represents the largest domestic use of maize in the U.S.A., at about 44% of total domestic use. This has resulted in a rapid increase in maize prices in the U.S.A. and similar upward pressure on other grains like wheat, and overall food commodity prices. Price of one metric ton of maize was about $80 in 2001, and had gone up to about $300 in 2011. Because U.S.A. is such a large part of the export market, price trend in the U.S.A. basically dictates world maize prices. Concerns have been raised that increased demand of grains for bioethanol production will lead to increased food insecurity in the poor nations which depend on grains for basic survival. Obviously more sustainable sources of renewable energy will be required to ensure global food security.

Sorghum and Millet

Overall world production of sorghum and millet is much lower than the 'big three'; for example, only 60 MMT of sorghum, and about 27 MMT of millet were produced in 2010. However, these grains play an especially important role in

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nutrition in parts of Africa and India, owing largely to their drought tolerance and other agronomic traits. Millets are especially drought tolerant and can perform well in areas receiving less than 350 mm rainfall (compared to about 700 mm minimum for maize). About 50% of sorghum and 80% of millet production is used for human consumption.

Like rice, sorghum and millet consumption is typically high in the regions where they are produced. For example, even though annual average per capita sorghum and millet consumption is about 8 kg in India, in the states where these crops are grown, per capita consumption exceeds 90 kg (*11*). In Burkina Faso and Sudan, per capita consumption of sorghum and millet is more than 80 kg, whereas the figure exceeds 90 kg in Niger (where millet accounts for 80% of cereal grain consumption) (*5*). In Nigeria, which is the leading world producer of sorghum (11.5 MMT in 2010), per capita sorghum consumption is about 50 kg. By contrast, even though U.S.A. is the second leading producer, hardly any sorghum makes it into human foods in the U.S.A., with most of the grain used for animal feed and export. However, growing evidence on potential health benefits of sorghum (*12–14*) have seen increased interest in their food application.

Sorghum and millet demand for human food use has been on the decline in India and Africa. This has been unfortunate, particularly in East and Southern Africa where sorghum fields have been largely replaced with maize, which has become the primary staple in these regions. Maize, unlike sorghum and millet, requires even moisture distribution throughout growing season and cannot withstand drought stress. This has contributed to reduced food security in these regions as subsistence farmers who rely on rainfall end up with practically no maize harvest during droughts. Other problems, like aflatoxin which is more prevalent in hot, high stress environments where sorghum and millets are traditionally grown, readily afflict maize when grown in these environments, and has resulted in significant deaths from consumption of contaminated maize (*15*).

Cereal Grains in Nutrition and Health

Cereal grains represent a contradiction of sort when it comes to nutrition and human health. In developing countries, malnutrition incidences, particularly related to protein, iron, zinc and vitamin A deficiencies, are highest in places with the highest per capita consumption of cereal grains. The two major reasons are that cereal grains are generally low in essential amino acids, particularly lysine; in addition, the refine grains most commonly consumed (e.g., polished rice) are very low in micro nutrients. In the developed countries, the refined grain products have been increasingly cited as a major contributor to obesity due to their high content of easily digestible carbohydrates (or 'carbs' in the diet lingo). As previously mentioned, the drop in wheat consumption in the U.S.A. in early 2000 (Figure 3), was attributed to the low 'carb' diet craze of that began in late 1990s. More recently, the maize-derived high fructose corn syrup (HFCS) has become the latest victim in the on-going search for a scapegoat for the ever expanding waistlines in the U.S.A..

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On the other hand, evidence suggests that populations in developing countries that consume certain whole grains more frequently, particularly sorghum and millet, have lower incidences of cancer and other chronic diseases than populations that primarily consume refined grains like wheat (*16*, *17*). In the developed world, health benefits of whole grain consumption have been extensively documented, particularly in the past 10 years. A Scopus® search for "whole and grain and health" as keywords revealed that of the 721 publications that showed up, 621 (86%) were published in the new millennium; a similar search of Web of Science® reveals that 91% of publications came in 2000 or later.

The tremendous number of scientific studies that have emerged in the past decade confirming benefits of whole grains against cardiovascular disease, cancer, diabetes, obesity, and a host of other chronic conditions, have resulted in growing popularity of whole grain products. In fact, according to the NPD Group market research (*18*) the American (U.S.A.) public now views whole grain as the single healthiest food item, ahead of broccoli. This has resulted in annual double digit growth in the whole grain product category demand since 2005. Even more encouraging is the fact that the demand is largely driven by the younger demographic (18-34 year old) who were previously averse to whole grain consumption. However, consumption of whole grain is generally still far below recommended intake. By some estimates, only 12-14% of the U.S. population consumes the recommended daily whole grain intake. Among the issues that will need to be considered in ensuring grains contribute maximally to overall health in the developing and developed worlds include addressing sensory appeal of whole grain products. Developing appropriate processing technologies will be important in producing products with proven health benefits that consumers can use on a long term basis.

Improving Impact of Cereal Grains on Human Health

Milling technologies that were industrialized in the 1800s made refined grain products affordable and accessible to the masses. These milling technologies efficiently remove the bran and germ of cereal grains where most of the dietary fiber, lipids, vitamins, minerals and phytochemicals are located (Table 3). Roller milling of wheat to obtain 70% extraction flour will generally reduce the levels of essential vitamins and minerals by 60 – 80%. The refined flour is mostly endosperm and contains 80 – 86% starch on a dry basis, with very low dietary fiber and mineral content (Table 3). A major advantage of the refined flour (and other refined grain products) is better keeping quality due to low lipid content. Whole grain products (e.g., brown rice) are prone to oxidation and quick onset of rancidity. Another huge advantage of refined grain products is their better sensory appeal due to the bright color, smooth texture, and mellow flavor, when compared to whole grain products.

Given the central role of cereal grains in human nutrition, technologies that resulted in widespread consumption of refined grain instead of whole grain stripped large populations of their primary source of essential nutrients, particularly vitamins and minerals (as well as dietary fiber and health promoting phytochemicals). In fact the discovery of a number of vitamins in the early

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20th century is directly attributable to consequences of deficiencies created by grain refining or improper processing; from beriberi link to vitamin B_1 (thiamine) deficiency and pellagra link to vitamin B_3 (niacin) deficiency. Even though voluntary enrichment of refined grain products has virtually eliminated micronutrient deficiencies in the developed world, major problems persist in the developing countries. Besides, the higher risk for chronic diseases associated with refined grain consumption necessitates intervention strategies that incorporate more whole grain components in daily diet. However, the near universal sensory appeal of refined grains among consumers worldwide presents significant challenges to food scientists in developing products that incorporate significant quantities of whole grain components while maintaining consumer acceptability.

Table 3. Proximate composition of various components of wheat

Component	*Grain*	*Bran*	*Germ*	*Endosperm*
Protein %	11 – 16	15 – 18	34	10 – 15
Fat %	2.3 – 3.5	4.5 – 5.7	12	< 1.0
Ash %	1.7 – 2.3	6.2 – 7.4	4.5	0.2 – 0.3
Dietary fiber %	13 – 15	40 – 45	15 - 18	1.1 – 2.3
Available carbohydrates %	70 – 76	15 – 18	35	80 – 85
Proportion of whole grain	**100%**	**12 – 16%**	**2.0 – 3.0%**	**80 – 85%**

Note: In general essential micronutrients including thiamin, riboflavin, niacin, iron, and zinc, are reduced by 60 – 80% in flour compared to whole grain.

Processing technologies are probably the most viable option to return people to consuming more whole grain components in their diet. Whole grain components that are rich in important micronutrients often contribute undesirable attributes in products, from reduced shelf life, to bitter taste and coarse texture contributed by bran phenolics, insoluble carbohydrates, and other fiber components. Technologies involving improved milling techniques to produce the right particle size of whole wheat flour, for example, have been recently developed and are currently in use to produce whole grain flours for various baking applications. Ingredients that mask bitter flavors are widely used to improve taste profile of various whole grain products. Textural attributes of products containing whole grain components have improved dramatically over the past two decades through careful use of selected additives and processing conditions. Newer technologies to utilize grain milling by-products to isolate active compounds (lipid alcohols, sterols, phenolics, feruloylated arabinoxylans, etc.) for specific food applications, or transform these components into forms that can be more easily used in foods without negatively impacting food acceptability are under investigation and development. Some of these are covered in the various chapters of the book.

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The expected growth in consumer demand for whole grain products will invariably inspire more research into developing products that meet consumer sensory expectations. Even though most of the whole grain-related research has focused on the developed world where incidences of obesity and over-nutrition are most apparent, ignoring the developing world would be an unfortunate mistake. The same approaches used to improve health attributes of food to help combat chronic disease in the developed world produce the exact same benefits in the developing world. The growing economies in the third world have produced a growing middle class that readily 'upgrades' to western diets as a reward for their improving economic status. Thus the incidences of obesity and associated disease are just as high among the middle class segment of developing countries as most developed countries. In addition, the poor majority in these countries have high incidences of malnourishment and nutrient deficiencies that improved palatability of whole grains components can go a long way in addressing.

It is rather unfortunate that investment in research to improve food nutritional quality which can contribute most meaningfully to prevention of chronic disease is often overlooked by most governments and other funding agencies in favor of seeking treatments for diseases once they occur. For example, the US government invests billions of dollars every year on biomedical research aimed at discovering causes and treatments for disease through National Institute of Health, National Science Foundation, and other non-obvious agencies like Department of Defense and Department of Agriculture, among others. By comparison, the total amount of money that goes into prevention research (including food science) is a drop in the ocean. Many governments and organizations are quick to advise the public on what they ought to eat to stay healthy. However, based on more than 100 years of evidence, people will not be persuaded to eat much of anything just because it is supposedly 'good for you.' A major obstacle with many potentially healthy products is that they do not meet consumer quality expectations. Addressing this more effectively will go a long way in ensuring food is a primary vehicle to prevent disease. The following chapters highlight some of the approaches currently under investigation.

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Chapter 2

Enhancing Micronutrient Content in Cereal Foods

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Cereal grains are generally good sources of micronutrients. Enhancing cereal foods as micronutrient sources is discussed in this chapter, focusing especially on folate and tocols. Possibilities for further increasing the content with conventional breeding vary among different micronutrients. Knowledge of the localization of the individual micronutrients could be utilized to produce fractions with the desired composition. Bioprocesses, germination and fermentation, offer further options to enhance, e.g., folate content.

Introduction

Cereal grains and thus cereal foods are important sources of micronutrients (i.e., vitamins and minerals) as well as phytochemicals with possible health benefits. The micronutrient and phytochemical content in grains may vary, however, because of genetic and environmental factors (*1*, *2*).

The health benefits of consuming whole grain products have been widely demonstrated (*3*–*5*). Many factors, including dietary fiber carbohydrates, micronutrients and phytochemicals, may contribute to this effect (*6*, *7*). Because whole grain flours contain more components that provide health benefits than white flours, incorporating whole grain flours, instead of white flours, in foods would provide benefits. However, whole grain flours also change products' sensory and technological properties, which limits increasing the use of these flours.

Processing grains for fractions with tailored composition and processing grains or their fractions with bioprocesses, e.g., germination and fermentation,

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provide options for enhancing micronutrient content in cereal foods (*8–10*). Technological and sensory properties may also be modified in bioprocessing, helping incorporate micronutrient-rich matrices in cereal foods (*11*).

Among micronutrients, the focus in this chapter will be on one of the B group vitamins, folate, and on fat-soluble vitamin E compounds, tocols. Other micronutrients are covered in less detail. Cereal products are important sources of folate. Folate occurs as different folate vitamers, reduced derivatives of folic acid (pteroyl-L-glutamic acid), which are largely bound to polyglutamates. Folate is actively studied for its role in preventing neural tube defects in developing embryos and for association with, e.g., cardiovascular diseases, cancer and cognitive functions (*12*). For example, in Finland, where mandatory fortification of flours with synthetic folic acid is not practiced as is done, for example, in the US, cereal foods still provide ca 35% of the total dietary intake (*13*). On the other hand, folate intakes often fall below recommendations in countries that do not practice mandatory fortification (*14*). Thus, naturally enhancing folate in cereal foods, consumed in big quantities by the whole population, is of interest. Cereal products are also important sources of tocols, i.e., tocopherols and tocotrienols (*15*, *16*). The special feature of tocols in cereal grains is the high proportion of tocotrienols. Currently, only α-tocopherol is taken into account as vitamin E (*17*). However, all tocopherols and tocotrienols are antioxidants and may have other significant roles as dietary components. Researchers have suggested that these roles include, e.g., preventing neurodegeneration and lowering serum cholesterol levels (*6*, *18*, *19*).

In this chapter, possibilities for enhancing micronutrient levels, especially those of folate and tocols, in cereal foods are introduced, with a focus on wheat products. Among the options for affecting micronutrients in cereal foods, selecting micronutrient-rich grains as raw materials, fractionating the grains into micronutrient-rich fractions as well as bioprocessing, i.e., germinating and fermenting, are evaluated.

Micronutrient Content in Cereal Grains

Effect of Genetic and Environmental Factors

Micronutrient content varies among cultivars of each cereal (*1*, *20*). To be able to choose micronutrient-rich cereal raw materials or utilize plant breeding to further increase the content, knowledge of diversity among cultivars and other genotypes is needed. However, comprehensive studies comparing genotypes in controlled experiments and studying the stability of the properties in different environmental conditions are scarce. The number of studied cultivars is often limited, and in controlled experiments, the samples often come from only one location and one growing year.

One of the most comprehensive studies on vitamins in wheat was conducted by Davis et al. (*21*). They studied several B vitamins in 231 wheat cultivars from 49 growing locations and partly from three growing years (in total 406 samples) showing marked variation in the vitamin content and that factors leading to significant variation differed for the studied vitamins. In 12 Australian wheats,

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each from three to five locations, the total folate content ranged from 799 to 1143 ng/g dm (dry matter) (*22*). In 10 rye genotypes, grown in the same location, the total folate content was 630–780 ng/g fw (fresh weight) (*23*), and the total tocol content was 39.9-54.3 μg/g fw (*24*). Batifoulier et al. (*25*) showed a more than 2-fold variation in the thiamine, riboflavin, and pyridoxine content of 49 wheat cultivars from one location and growing year. These example studies indicate genetic variation in vitamin content. Marked variation in mineral content has also been shown among genotypes (*1*, *2*).

However, environmental factors also cause variation in micronutrient content, and the impact of genetic and environmental factors should be separated to see the real potential for conventional breeding. A big European project, Healthgrain (*20*, *26*), provided information on genetic and environmental factors. First, 200 cereal genotypes were screened for dietary fiber and various bioactive components (*20*). All the genotypes were grown in highly controlled conditions in the same location in Europe, Hungary. Most of the samples represented wheat; 130 winter and 20 spring wheat genotypes were included. The number of genotypes for other cereals (other wheats, rye, barley, oats) was much smaller, 5 or 10 genotypes. Environmental factors were studied by comparing three growing years in the same location in Hungary and by comparing four growing locations, Hungary, France, the United Kingdom and Poland, in the same year (*26*). In these comparisons, 26 wheat genotypes were included.

Taking folate and tocols as examples, all the screened cereals could be regarded as good folate sources and moderately good tocol sources although some differences were seen in the average content (*20*) (Figure 1). The highest mean total folate content was measured in durum wheat, early cultivated dicoccum (tetraploid emmer) and rye and the highest total tocol content in barley and rye. Because the number of samples for cereals other than wheat was small, this comparison is, however, only indicative. The results also showed that each cereal's genotypes varied. The total folate content showed 2-fold variation (*27*) and those of total tocols 2.9-fold variation (*28*) in winter and spring wheat. In winter wheat, the folate and tocol content of the 130 genotypes varied at 364–774 ng/g and 27.6–79.7 μg/g dm, respectively.

The results also showed marked variation among growing years. The average total folate content ranged from 591 ng/g dw in 2005 to 728 ng/g dw in 2007. The average content was thus 23% higher in 2007 than in 2005 (*29*). When the results of the four locations were compared, a variation in the folate content was again clear. The average folate content of the samples from Hungary was 44% higher than in those from Poland. The total variation among all the samples was from 367 to 889 ng/g dm, which shows marked variation. The main folate vitamer was 5-formyltetrahydrofolate, accounting for 35% of total folate, and two other formyl derivatives accounted together for a further 30%. Variation in the proportions of 5-methyltetrahydrofolate and 5-formyltetrahydrofolate mainly explained the variation in the total folate content; the contribution of 5-methyltetrahydrofolate was higher and that of 5-formyltetrahydrofolate lower in samples with higher total folate content (*29*). Further, genotypes with a high or low folate content and with a narrow or broad range were identified. In terms of the tocol content, some of the genotypes were also more sensitive to the impact

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of the environment whereas others were relatively stable. The total range among all the samples was 30.8–87.9 μg/g dm (*30*). β-Tocotrienol was the major tocol followed by α-tocopherol. The other tocols occurring in significant amounts in all the samples were α-tocotrienol and β-tocopherol. The β-tocotrienol content varied the most whereas the α-tocopherol content was the most stable. Strong positive correlations with the mean temperature between heading and harvest were shown in the folate and tocol content, whereas the folate content also showed negative correlation with total precipitation between heading and harvest (*26*). The stability of the levels in different environments is of practical relevance in terms of providing constant levels of micronutrients in cereal foods.

The published studies thus show clear variation in the micronutrients of currently available cultivars. Some studies also indicate that there are differences among cultivars in the stability of the content, thus enabling identification of cultivars with high and fairly stable content of the micronutrient of interest.

Possibilities for Increasing Micronutrient Content in Grains

The results of the Healthgrain project also showed that the role of the genetic factor differed for the studied components in wheat (*26*). The ratio of the genotypic variation to the total variance was high (0.77) for tocols showing high heritability. This high ratio indicates that tocol content is a realistic target for selection in plant breeding. However, the much lower ratio of the genetic to total variance for folates (0.24) indicates that stable increases in folate content will not be readily achieved with breeding. Thus, the possibility for obtaining significant effects with conventional breeding differ among micronutrients.

The content of tocols, folate and many other components in the studied wheat genotypes (the results of the first screening study by Ward et al. (*20*) and of the study for environmental variation by Shewry et al. (*26*)) were also analyzed to study whether the content had decreased because of intensive breeding. No decreases in the content of any groups of bioactive compounds were observed (*31*). The study thus showed that the bioactive component content was not related to the age or origin of the genotypes; some of the genotypes with the highest content were modern high-yielding cultivars.

Biofortification using metabolic engineering could significantly enhance the micronutrient content. Folate was fortified in rice seeds by overexpressing two *Arabidopsis thaliana* genes of the pterin and para-aminobenzoate branches of the folate biosynthetic pathway from a single focus (*32*). A maximal enhancement as high as 100-fold above the wild type was achieved. A portion of 100 g of polished raw grains would provide up to four times adults' daily folate requirements. Further difference was seen in the level of folates bound as polyglutamates: ca 2.6–14% in transgenic lines versus 50% in wild genotype. The lower occurrence as polyglutamate could improve bioavailability. The authors report that the maximum level achieved, 17.23 μg/g fw, was the highest folate content reported for a plant species at that time. Enough is known about folate biosynthesis to enhance folate content in other crops, such as wheat. Folate biofortification of food crops would thus be a feasible complementary strategy if the methods were accepted for use (*33–35*).

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Based on the published studies, the role of genetic factors seems to differ in the case of specific nutrients affecting possibilities for further enhancing cereal raw materials as micronutrient sources with conventional plant breeding. Biofortification would efficiently enhance micronutrient content.

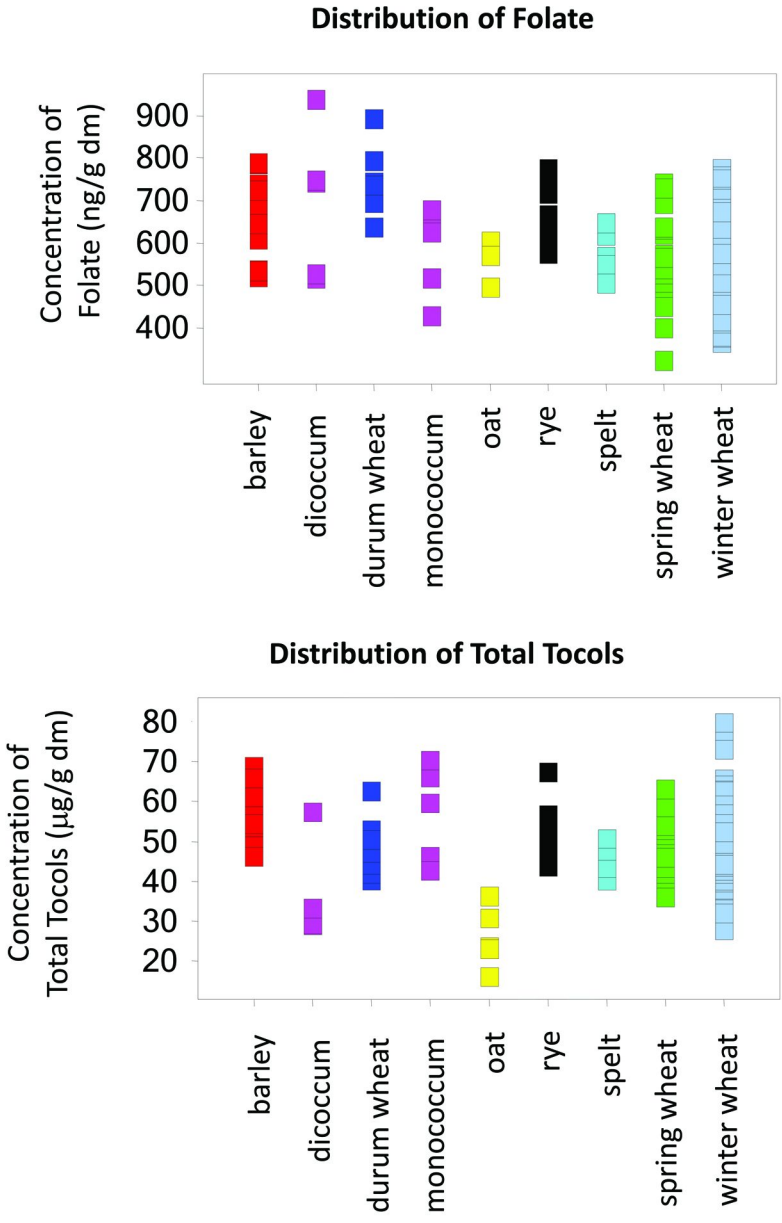

Figure 1. Variation in folate and tocol content in different cereals. (Reproduced with permission from reference (20). Copyright 2008 American Chemical Society.)

Micronutrient-Rich Grain Fraction

Micronutrients are known to be concentrated in the outer layers of grains although detailed localization of the individual components is not always well-known (*1*, *10*). Therefore, whole grain flours are clearly better micronutrient sources than flours with low extraction rates. Further, research on developing the fractionation processes gives interesting options for further enhancing micronutrients in cereal foods.

Flours and Other Milling Fractions

Based on earlier research, Nelson et al. (*36*) concluded that 60% of the minerals in wheat grains are located in the aleurone layer and 20% in the endosperm. They further concluded that scutellum contributes 8%, pericarp and testa 7% and germ 4% to the total amount of minerals. Wheat flour with an extraction rate of 66% contained 28%, 29%, 33% and 52% of zinc, iron, copper and calcium of the whole grains, respectively (*37*, *38*). The contents of six B vitamins were lower the lower the extraction rate in wheat, rye, barley, sorghum and rice flours (*39*). In wheat flour with an extraction rate of 66%, 39% of riboflavin was retained, whereas the corresponding figures for thiamine, pyridoxine, biotin, niacin and folate were 24%, 17%, 22%, 13% and 11%, respectively (*39*). Thus, niacin and folate were strongly affected by milling whereas riboflavin was more evenly distributed. A later study reported that 43%, 67% and 20% of thiamine, riboflavine and pyridoxine were recovered in white wheat flour after milling (*40*). Michalska et al. (*41*) reported that in rye flours with different extraction rates the total tocol content and the tocotrienol/tocopherol ratio were higher the higher the extraction rate. In general, the vitamin and mineral content in flours has been shown to be correlated with the ash content. The extraction rate is thus probably the most important factor controlling the levels of B vitamins, tocols and minerals in flour (*1*, *10*, *37*).

Certain commonly produced grain fractions could be used to enhance the micronutrients. Among common wheat fractions, especially germ and bran are rich in micronutrients (Table I). In the studies reviewed in Table I, the total folate and tocol content in wheat bran was 704–1600 ng/g and 76–106 μg/g dm, respectively. The folate content in wheat bran (1600 ng/g dm) was 4-fold compared to that in flour (extraction rate 74.7%) and 2.5-fold higher than in grains (*22*). In commercial rye and wheat brans, the folate and tocol content was 400–1000 ng/g and 78–131 μg/g dm, respectively (*42*). To study the distribution of various bioactive compounds in rye grains, the grains were milled with a roller mill to give four streams: bran, shorts and two flours (milling fraction from reduction rolls and milling fraction from brake rolls). Part of the bran was further cleaned with roller milling. The outer bran fraction contained 1.6–2.1 times more folate, tocols and several other bioactive components than whole meal rye flour (*43*). The bran fraction contained 10-fold more folate and tocols than the flours. However, the germ fraction would be still a better folate as well as tocol source. The total folate content in the wheat germ was more than 3-fold compared to that in bran (2400 ng/g vs 700 ng/g dm) (*44*). Correspondingly, wheat germ had

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more than 3-fold tocol content compared with bran (*44*, *45*). Holasova et al. (*46*) reported that the total tocol content in wheat grain, germ and bran was 44, 181 and 76 µg/g dm. The tocol content in rice germ, bran and endosperm was 425, 238 and 5 µg/g and that of the same fractions of barley 134, 117 and 12 µg/g dm (*47*). Wheat germ and endosperm contained 257 and 17 µg/g and corn germ and endosperm 463 and 15 µg/g dm of total tocols, respectively. Including the germ into the flour would thus improve, for example, the total folate and tocol content of bread. However, the germ should first be stabilized to avoid oxidative rancidity (*48*). Adding substantial amounts of bran is also challenging because of its influence on technological and sensory properties. In addition to the total vitamin content, the vitamer compositions may differ in different parts of the kernel. Among tocols, α-tocopherol in particular is localized in the germ. Therefore, wheat germ oil is one of the best sources of vitamin E (*49*). In wheat flour, β-tocotrienol is the major tocol (*46*).

Table I. Folate (ng/g of Dry Matter) and Tocol Content (µg/g of Dry Matter) in Wheat Grains, Bran, and Germ

Fraction	*Total folate*[a]	*Total tocols*[a]	*α-tocopherol*[a]
Wheat grains or whole meal flour	220–650 (*22*, *44*, *50*)	44–48 (*44–46*)	9.8–13.5 (*44–46*)
Bran	704–1600 (*22*, *42*, *44*)	76–106 (*42*, *44–46*)	3–16 (*42*, *44–46*)
Germ	2396 (*44*)	181–319 (*44–47*)	104–221 (*44–47*)

[a] references in parenthesis

By-products of common processes may be good micronutrient sources. For example, the total tocol content of barley pearling flour, representing 20% of the kernel weight, was 2.9-fold compared to that of grains (*51*). High total tocol content was also found in fifth middling, red dog and reduction shorts. In a later study, the pearling flour of barley was the richest barley milling fraction containing 240 µg/g fw of total tocols (*47*). The pearling fraction of barley was also especially rich in folate with more than 2-fold content compared with grains (*52*). These fractions, however, are not commonly used as food.

Processes To Separate Micronutrient-Rich Fractions

Good knowledge of the localization of the various micronutrients in grains helps to develop fractionation processes further. Dry fractionation technologies can be developed to make better use of different grain parts and to produce new flours and ingredients with optimized technological and nutritional quality (*53*). Bran, a composite multi-layered material (outer pericarp, inner pericarp, testa, hyaline layer, aleurone layer, attached starchy endosperm residues), could

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be separated into various fractions (*54*). One of the processes is debranning, which removes bran layers sequently by friction and abrasion (*55*). Processes based on debranning may also be used to remove the most external bran layers before milling and thus to obtain bran, which is richer in aleurone cells (*55*, *56*). Different grain pretreatments, improved mechanical tissue dissociation and particle reduction, and improved particle sorting and classification could be utilized to produce fractions with the desired composition (*57*).

For example, a process was developed and patented to separate the nutritionally rich aleurone layer from other bran layers (*58*, *59*). In the patented method, a special mill separates the aleurone layer from the pericarp layer, and then several purification steps isolate the aleurone layer. Some fractions are further reduced by micromilling. The folate and α-tocopherol content in the product was 1580 ng/g and 12 μg/g dm, respectively (*59*). Substituting 20% of white flours with the aleurone fraction increased pyridoxine and minerals to the level of whole grain flour and that of niacin to the 4-fold level (*60*). Fenech et al. (*61*) showed that the folate in the aleurone fraction was bioavailable. They concluded that using wheat aleurone flour in the diet could be considered as an alternative strategy for increasing folate intake in the general population. The total folate content in the preparation (containing aleurone cells and germ) was reported to be as high as 5150 ng/g fw whereas the content in whole grain flour was 940 ng/g fw (*61*).

As part of the Healthgrain project, new processes were studied. The effects of the grinding temperature (ambient, cryogenic) and the potential for electrostatic separation were investigated to obtain purer fractions. Ultrafine grinding followed by electrostatic separation allowed fractions with very different particle size and composition to be produced. Fractions rich in aleurone (i.e., rich in minerals and vitamins) and rich in the most outer layers (i.e., rich in fibers) were separated (*57*). Further possibilities for improving the separation of the aleurone cell content (folates, minerals) were identified.

Germination as a Bioprocess for Increasing Micronutrient Levels

Germination or malting, i.e., controlled germination, may be used to pretreat cereal grains to modify their structure, composition and flavor (*62*). In germination, the biosynthetic potential of grains is activated leading to modification of the composition and to biosynthesis of bioactive components. Germinated grains may be further processed, e.g., with fermentation. Beneficial changes obtained either by germination alone or by the two processes together may give new possibilities for incorporating whole grains with increased levels of components that are beneficial to health into foods (*11*). Germination has been shown to increase folate content in grains (*43*, *62*, *63*). During germination, folate synthesis is accelerated because of the increased demand for C1-units in the developing seedlings. Some studies also report that the content of other B vitamins increased (*64*, *65*). The tocol levels were similar in native and germinated grains whereas a 1.5-fold level was measured in rootlets (*62*).

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During germination of rye grains, the folate content increased by 1.7–3.8-fold, depending on the germination temperature, reaching a maximum content of 2500 ng/g dm (*63*). Hypocotylar roots were especially rich in folate containing 6000–11800 ng/g dm of folate. That contributed 30–50% to the content in the germinated grains. The increase was therefore lower, 1.8–2.5-fold, when the roots were removed in another experiment. The content in roots was 10-fold and 19-fold higher than in germinated and native grains, respectively (*62*, *63*). Thermal processing, which may be needed after germination, destroys part of folate. However, germinated and dried rye grains (without roots) contained more folate than native grains after all tested thermal treatments, i.e., extrusion, autoclaving and puffing, IR and roasting (*63*). Statistical experimental design and response surface modeling were used to optimize germination parameters. The optimum germination temperature for folate synthesis was 14–16°C (*62*). When rye grains were germinated in commercial malting equipment in darkness at 18°C for 6 days, the folate content increased from 610 ng/g fw in grains to 2170 ng/g fw in the germinated grains with rootlets (*66*). The malting conditions used in this study for rye were comparable to those used for industrial malting of barley. An increase of a comparable level was measured by Koehler et al. (*67*) in wheat grains. The maximum increase (3.6-fold) was found after 102 h germination at 20°C (Figure 2). Industrially malted rye, wheat and oats were also fairly rich in folate, containing 1400–3300, 1400 and 700 ng/g fw of folate. In the barley malting process for producing beer, the folate levels increased 2- to 3-fold up to the content of 2000–3000 ng/g fw (*8*). Germination also changes folate vitamer composition. In grains, formylated vitamers dominate whereas in germinated grains proportions of 5-methyltetrahydrofolate and tetrahydrolate were shown to be significantly higher (*63*, *67*) (Figure 2).

A number of studies have demonstrated that using malting as a pretreatment leads to enhanced folate levels in the final cereal foods. Malted rye grains were used to produce muesli with 1900 ng/g fw of folate for a human intervention study (*68*). Enhanced folate content in malted flour may markedly contribute to the folate content of the bread. In the study by Arcot et al. (*22*), malt flour contained 1580 ng/g dm of folate that was 4-fold higher than in white flour. Germination resulted in 4-fold higher folate content in wheat grains (*69*). Thus, substituting 50% of flours with germinated wheat flour in baladi bread baking markedly increased the folate content in bread while the bread was acceptable with respect to color and layer separation. Malts could be used in addition to baking in breakfast cereals, biscuits and snacks, e.g., to increase their nutritional properties (*62*).

Fermentation as a Means to Increase Micronutrient Content

Fermentation can be used as part of the bread-baking process and as a process to modify the cereal matrix, e.g., grains or bran, for further processing (*9*, *11*, *70*). Sourdough fermentation could improve the palatability and processibility of whole meal flour and bran. This process may thus facilitate incorporating dietary fiber-rich cereal materials in bread and other products. Furthermore, other products, such as, e.g., healthy snacks, biscuits, and other types of convenience foods, could

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be produced utilizing fermented cereal ingredients. Fermentation has also been shown to increase folate and other B vitamin content.

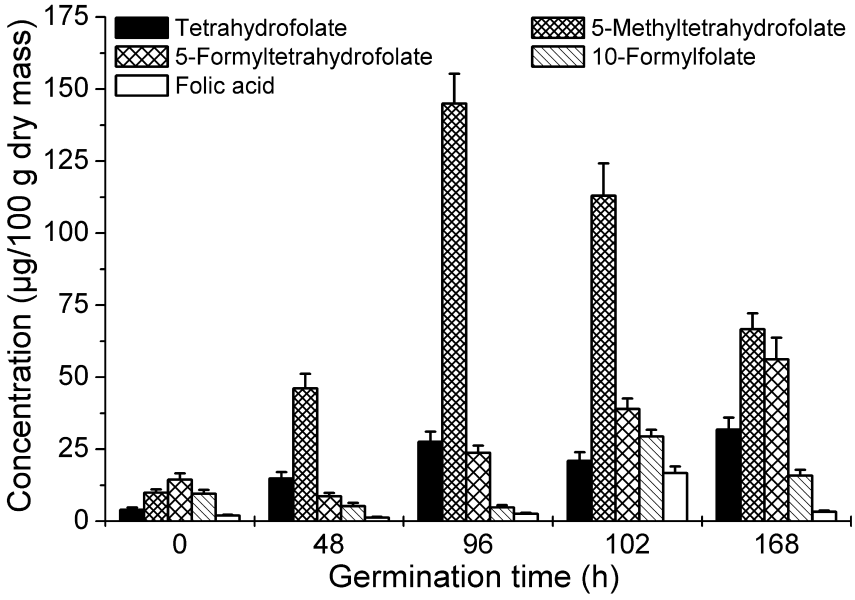

Figure 2. Folate vitamer distribution in wheat grains germinated for different times at 20°C. (Reproduced with permission from reference (67). Copyright 2007 American Chemical Society.)

General Changes in Composition and Micronutrients

In fermentation, the cereal matrix is modified by the metabolic activity of microbes. Fermentation induces structural breakdown of cereal cell walls that may lead to liberation and/or synthesis of various bioactive compounds (*11*). Furthermore, yeasts and bacteria can synthesize, for example, B vitamins. Mineral bioavailability may also be improved because of the breakdown of phytate, which occurs in the highest concentrations in the micronutrient-rich aleurone layer (*71*). However, levels of unstable compounds, e.g., tocols, may also decrease in long fermentation (*62*). Sourdough fermentation also stabilized wheat germ (*72*).

Especially promising results for the potential of enhancing B vitamin have been obtained in folate research (*22*, *23*, *43*, *66*, *70*). Endogenous grain microbes and starter microbes, especially yeasts, synthesize folate in cereal fermentation (*63*, *73–75*). Some bacteria isolated from oat bran had higher intracellular folate content than *Saccharomyces cerevisiae*, which is known to be a good folate producer (*75*).

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Fermentation as Part of the Baking Process

The sourdough fermentation step of the rye bread-baking process doubled the folate content when the fermentation was done with baker's yeast and *Lactobacillus* cultures for 22 h at 30°C whereas the tocol content decreased during fermentation (*43*). Kariluoto et al. (*23*) compared three methods in rye bread baking: 1) bread leavened with yeast (*S. cerevisiae*), 2) traditional sourdough fermentation with *S. cerevisiae* and lactic acid bacteria and 3) sourdough fermentation without added yeast. The folate content increased during the traditional sourdough fermentation and fermentation with yeast, and the increase was associated mainly with the growth of the yeast. Yeast was able to compensate for folate losses in baking with its high folate content and by synthesizing folate during fermentation. When rye bread was baked using lactic acid bacteria fermentation without yeast, the bread contained 31% less folate than that with yeast. In more detail, in sourdough fermentation experiments with added yeast, the increase in folate content was 54% and 128% depending on the rye raw material (Figure 3). Three methods were also compared in wheat baking: 1) sponge dough method, 2) straight-dough method and 3) straight dough with baking powder. Wheat bread baked with the sponge-dough or straight-dough method had 2.5-fold folate content compared with bread leavened with baking powder. Marked differences have been shown in the folate production capacity of different *S. cerevisiae* strains (*63*, *73*, *74*). Folate production of two *S. cerevisiae* strains, one *Candida milleri* strain as well as one *Torulaspora delbrueckii* strain were compared as monocultures in sterile and non-sterile rye flour and water. *C. milleri*, a typical sourdough yeast, was as good a folate producer as the better *S. cerevisiae* strain. Hjortmo et al. (*74*) showed later that by choosing the yeast strain and cultivation procedure folate content in bread was increased 3- to 5-fold compared to bread made with commercial baker's yeast. Several typical sourdough lactic acid bacteria did not produce folate (*63*).

Several other researchers have also reported marked increases in folate levels in the fermentation step of the baking process. When the total endogenous folate content in wheat flour was 480 ng/g dm, the folate content of sponge, proofed dough and bread was 830, 700 and 560 ng/g dm, respectively (*77*). Folate production in fermentation thus more than compensated folate losses in baking. Arcot et al. (*22*) reported that the folate content increased during the fermentation step by 232%. An increase in folate content was also reported by Guiska and Majewska (*78*). Changes in thiamine, riboflavin and pyridoxine content in wheat bread baking were studied by Batifoulier et al. (*40*). The most significant changes were seen in the riboflavin content. The total riboflavin content was 350% higher in dough after long fermentation with yeast than in white flours. When whole wheat flour was used, the corresponding increase was 190%.

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Fermentation as Pretreatment

Katina et al. (*66*) studied fermentation of flour from native or germinated rye comparing five different types of fermentation: 1) *Lactobacillus plantarum* (homofermentative), 2) *Lb. brevis* (heterofermentative), 3) *S. cerevisiae* (baker's yeast fermentation), 4) *S. cervisiae+Lb. plantarum+Lb. brevis* (combination starter, mixed fermentation) and 5) spontaneous fermentation (no added microbes). The flours were mixed with tap water +microbe and fermented for 20 h at 30°C. Yeast fermentation (either pure or mixed culture) of native grains led to ca. 2-fold folate content and fermentation after germination to ca. 8-fold content compared with the original grains. The folate levels did not change during lactic acid or spontaneous fermentation of native or germinated rye meal.

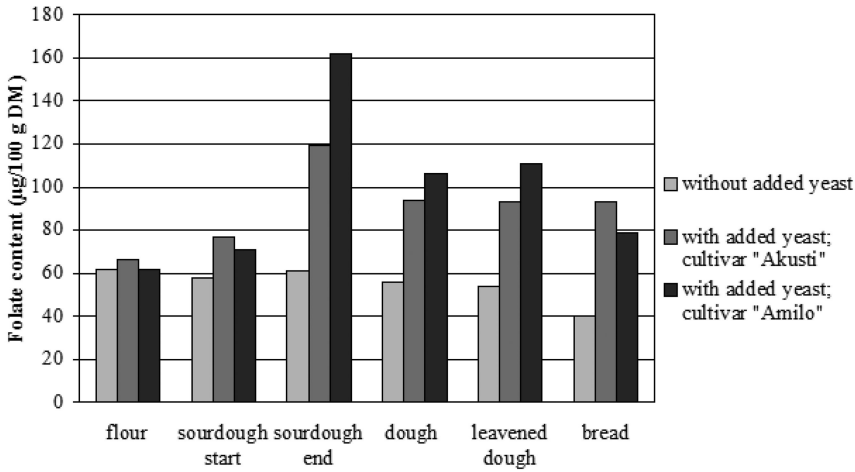

Figure 3. Total folate content during sourdough baking of rye. (Reproduced with permission from reference (76). Copyright 2008 EKT Series, University of Helsinki.)

Fermentation of rye bran from native and peeled (reduced the microbial load) grains markedly increased the folate content (*70*). The folate content increased 2.3-fold during yeast fermentation of flour from peeled grains at 35°C for 20 h and 1.9-fold in fermentation of flours from native grains at 28°C for 20 h. The increase was mainly due to the folate produced by yeast. However, yeast growth could not totally explain the increase. The highest folate level was obtained when the growth of endogenous lactic acid bacteria was pronounced, which may indicate that endogenous bacteria have a supportive role.

Conclusions

Micronutrient content in grains varies because of genetic and environmental factors. Based on recent studies, cultivars with constantly higher folate and tocol content may be separated; folate and tocols were chosen as the micronutrients

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of focus in this chapter. The possibility of increasing the content further with conventional breeding, however, seems to vary among different micronutrients. Using whole grain flour or fractions rich in micronutrients are efficient means for enhancing micronutrient content in cereal foods. Knowledge of the localization of individual micronutrients could be utilized to further develop fractionation processes to give fractions with the desired composition. Bioprocesses, germination and malting, offer further options for enhancing the B vitamin content. Baking experiments and other fermentation experiments have shown that fermentation especially using *S. cerevisiae* increases folate content. Further optimizing the fermentation processes may enhance the folate content even more.

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Chapter 3

Effects of Processing on Antioxidant Phenolics of Cereal and Legume Grains

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A wide variety of foods are processed from cereals and legumes using different processing methods. Food processing does impact on quality aspects such as sensory and nutritional. Various chemical reactions, physical and biological processes are set in motion during food processing. The phytochemical quality of foods as affected by processing becomes important in this regard. Phenolic compounds are a significant group of phytochemicals in cereals and legumes. They possess bioactive properties such as antioxidant activity and offer potential health benefits. Processing of cereals and legumes may enhance or reduce levels of phenolic compounds in foods and this has implications for their bioactive properties and potential health benefits they can offer.

Introduction

Cereals and legumes form a very important part of the diets of people around the world. They are sources of important macro- and micro-nutrients and so play a crucial role in human nutrition. Food processing as applied to cereals and legumes seeks to transform the raw grains into finished products for consumption by humans or animals. These processed foods should be of good sensory and nutritional quality. As would be expected, processing methods have an influence on the chemical constituents and physical properties of a food. The quality of processed foods is therefore of paramount importance.

Apart from the well known nutrients, cereals and legumes are also sources of phytochemicals which are non-nutritive plant chemicals that have potential health

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benefits. Phenolic compounds form an important group of such phytochemicals. Phenolic compounds possess bioactive properties such as antioxidant activity and ability to stimulate the body's natural detoxification enzyme systems and therefore offer some potential health benefits such as prevention of diseases like cancer, hypertension and heart disease. As a result, phenolic compounds in cereals and legumes are the subject of extensive research. An important area of research into phenolic compounds relates to how processing affects their levels in cereals and legumes and their resultant bioactive properties.

This chapter gives a brief overview of the chemistry of phenolic compounds in cereals and legumes and their significance in terms of the potential health benefits they offer. Some of the major processing methods applied to cereals and legumes and how they impact on levels of phenolics and their bioactive properties are then discussed.

Chemistry of Phenolic Compounds in Grains and Their Importance from a Health Perspective

Phenolic compounds may be defined as substances that possess an aromatic ring bearing one or more hydroxyl (OH) substituents (*1*). They are secondary metabolites produced by plants especially under conditions of stress such as in response to injury, UV radiation and infection (*2*). Phenolic compounds are involved in defense against UV radiation, aggression by pathogens, pests and oxidative stress (*3*, *4*). Various reviews are available on phenolic compounds, their chemistry and analysis, content in foods and nutritional significance (*2*, *5–9*). Phenolics are perhaps the most abundant group of compounds known in plants and it has been suggested that there are more than 8000 known phenolic structures (*10*). A simple and convenient way of classifying them is into phenolic acids, flavonoids and tannins, in increasing order of molecular weight.

Phenolic acids are derivatives of benzoic acid (e.g. gallic, protocatechuic, p-hydroxybenzoic acids) or cinnamic acid (e.g. caffeic, ferulic, p-coumaric acids) (Figure 1). In cereals, phenolic acids exist mostly in bound forms, esterified to arabinoxylans and also forming bridges between chains of hemicelluloses (*6*). The most abundant phenolic acid in cereal grains such as maize (*11*), sorghum (*12*) wheat (*13*) and rye (*14*) is ferulic acid. Ferulic acid is mainly concentrated in the outer parts of the grain, chiefly in the aleurone layer and the pericarp (*6*). Phenolic acids are reported to be concentrated in the cotyledon in legumes such as the common bean (*15*), lentils (*16*) and soybean (*17*).

Flavonoids consist of a 3-ring system (termed A, B and C) (Figure 2) and have been described as having a benzopyran nucleus (rings A and C) with an aromatic substituent (ring B) attached to the C ring (*1*). Groups of flavonoids include flavanones, flavonols, flavones, isoflavones, flavans and anthocyanins (Figure 3). It is reported that the anthocyanins of sorghum known as 3-deoxyanthocyanins, are unique in that they do not contain the hydroxyl group in position 3 on the C ring (*8*). Soybeans are well known as important sources of isoflavones.

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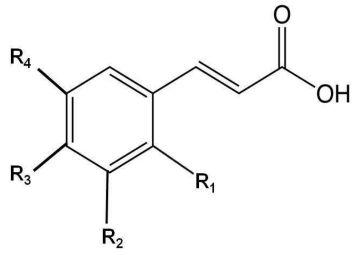
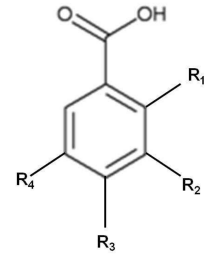


A. Cinnamic acid



B. Benzoic acid

Cinnamic acid derivatives	**R_1**	**R_2**	**R_3**	**R_4**
p-Coumaric acid	H	H	OH	H
Caffeic acid	H	H	OH	OH
Ferulic acid	H	H	OH	OCH_3
Sinapic acid	H	OCH_3	OH	OCH_3

Benzoic acid derivatives	**R_1**	**R_2**	**R_3**	**R_4**
p-Hydroxybenzoic acid	H	H	OH	H
Vanillic acid	H	OCH_3	OH	H
Gallic acid	H	OH	OH	OH
Syringic Acid	H	OCH_3	OH	OCH_3
Protocatechuic acid	H	OH	OH	H

Figure 1. Chemical structure and substitution pattern of representative phenolic acids found in cereal and legume grains.

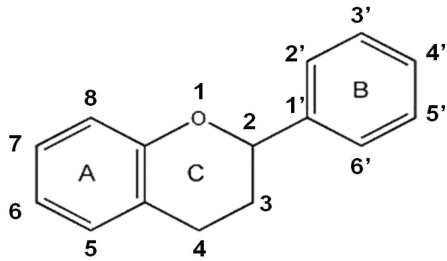


Figure 2. Generic structure of a flavonoid showing A, B and C rings.

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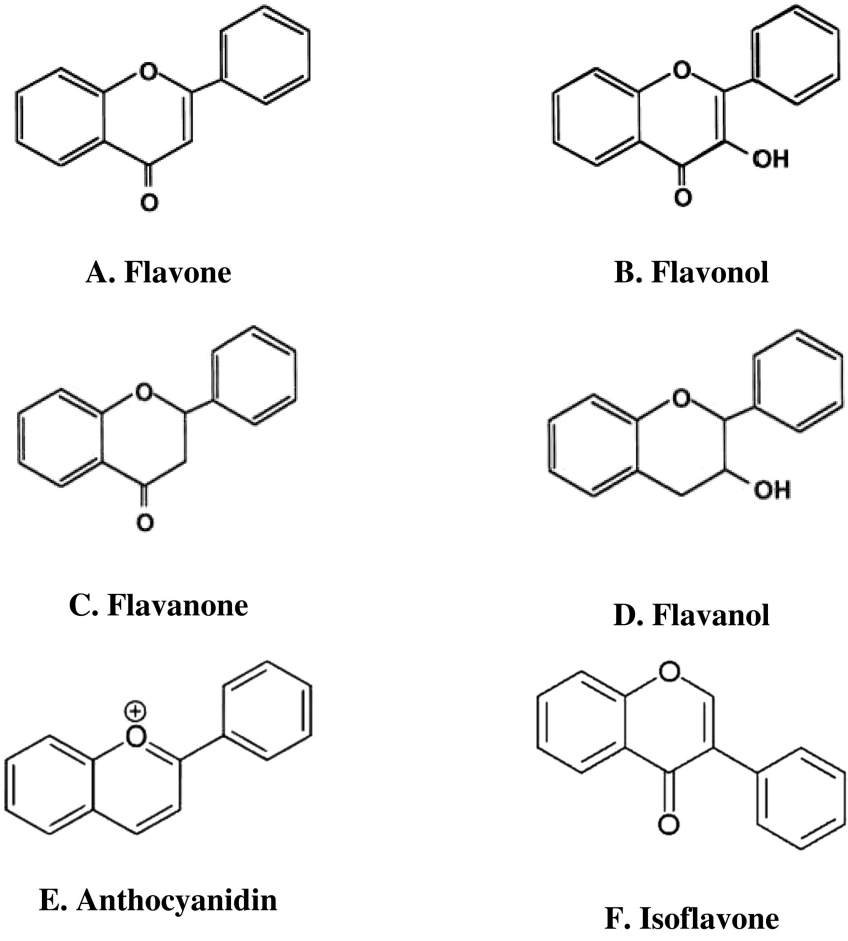

Figure 3. Structures of flavonoid subclasses.

Tannins occur either as hydrolysable or condensed tannins. Hydrolysable tannins are compounds in which a phenolic acid such as gallic acid is esterified to sugars such as glucose (Figure 4). On the other hand, condensed tannins are polymers of flavan-3-ol units linked by carbon-carbon bonds between the flavonol sub-units (*18*) (Figure 5). Cereals and legumes are not noted as important sources of hydrolysable tannins. The presence of condensed tannins in cereals appears to be restricted to a few, namely sorghum, finger millet (*19*) and barley.

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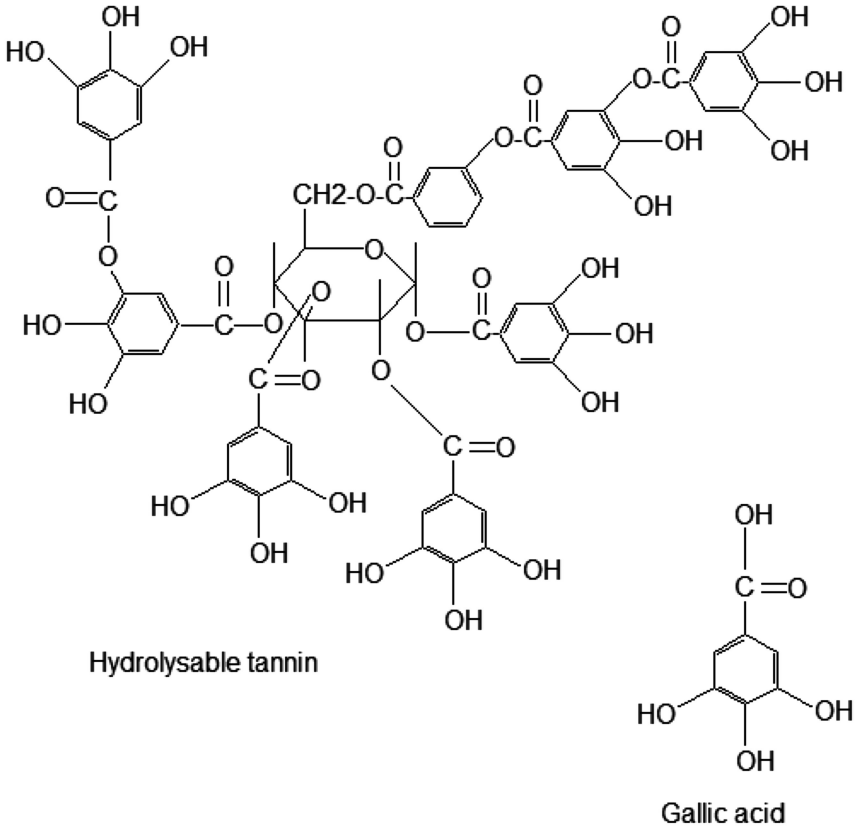

Figure 4. Structure of a hydrolyzable tannin.

Phenolic compounds (especially tannins) have been regarded as anti-nutritional factors due to their ability to form complexes with dietary proteins and minerals and digestive enzymes (*5*). However, there is now increasing focus on positive aspects of phenolics due to their ability to act as antioxidants which may offer potential health benefits such as prevention of diseases such as cancer and cardiovascular disease. Some biological activities associated with isoflavones for instance, include reduction in osteoporosis, prevention of cancer and cardiovascular disease and treatment of menopausal symptoms (*20*).

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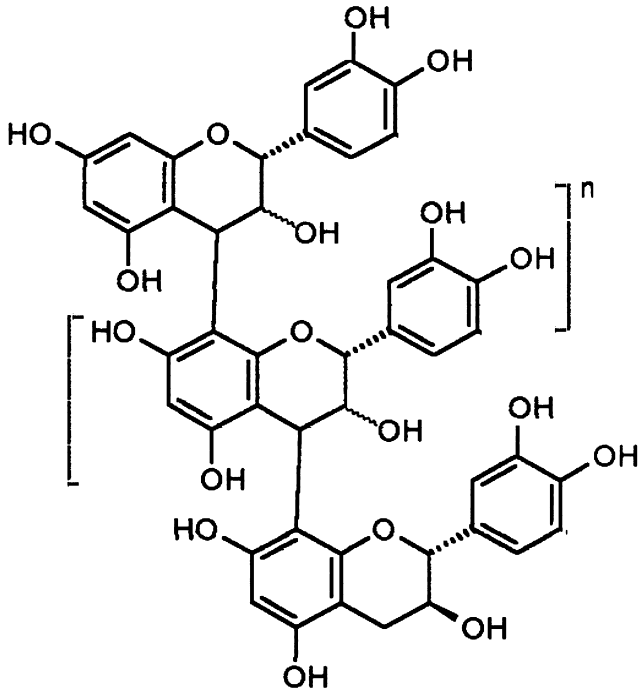

Figure 5. Structure of condensed tannin; n = 1 to > 10.

Effect of Processing on Phenolic Phytochemicals in Cereal and Legume Grains

A wide range of food processing methods are used to process cereal and legume grains into a wide variety of food products around the world. Such products range from porridges, breakfast cereals, alcoholic and non-alcoholic beverages, sauces to various types of baked goods. In this section, a few of some of the most important food processing methods and their effect on phenolic phytochemicals and their bioactive properties will be discussed. Table I provides a summary of some of the effects of processing on phenolics and antioxidant properties of various cereals and legume grains. This is by no means an exhaustive list. These are few examples to illustrate the wide ranging effects of processing on phenolic compounds and antioxidant properties of cereals and legumes.

Decortication or Dehulling

Decortication or dehulling refers to the operation where the outer layers of the grain (essentially the pericarp) are removed. In grains, this is invariably a component of the milling process. For cereals such as wheat and rye, the presence of a deep ventral furrow allows for the kernel to be broken open and the endosperm is then scraped from the bran. Milling technology allows for the

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production of flours of different extraction rates with varying extents of removal of the bran. Sorghum kernels are usually decorticated by abrading the outer layers off leaving clean endosperm which can then be milled into flour (*56*). Dry and wet dehulling methods have been described for legumes such as cowpea (*57*). Removal of the pericarp during the decortication or milling process is desirable because the pericarp is hard in texture and generally unpalatable. Decortication is conducted in the production of tannin sorghum food products in Africa as it reduces astringency, improves digestibility and produces lighter colored products (*56*).

In cereal (*58–60*) and legume grains (*15*, *17*, *61*), phenolic compounds are concentrated in their outer layers (pericarp, testa or seed coat). Therefore the decortication process reduces phenolic content and subsequently, antioxidant activity of the flours produced. In general, the extent of reduction in phenolic content increases in direct proportion to the extent of removal of the outer layers of the grain during decortication.

Various workers have reported on reduction in levels of phenolic compounds in cereals and legumes as a result of decortication and the following are just a few examples. In sorghum for instance, Youssef et al. (1988) (*21*) reported reduction in phenolics of the order of 70-85% due to decortication to 70% extraction rate. Dlamini et al. (2007) (*24*) observed reductions of 33-77% in sorghums of 70-81% extraction rates. Chiremba et al. (2009) (*25*) also found that decortication of sorghum to 70% extraction rate reduced total phenolic content by 43-66%. Similar results have been reported for pearl millet (*27*, *62*) and finger millet (*26*). Decortication reduced total phenolics and antioxidant activity by about 80% in lentils and 30% in yellow peas (*63*).

Although decortication may be desirable, it is clear that due to the loss of antioxidant phenolics, it may be a demerit from an antioxidant standpoint. There may be a need to find a balance between extensive decortication which reduces the levels of phenolics drastically and possibly compromises on potential health benefits, and optimal extent of decortication without affecting sensory quality of grain foods adversely. In this regard, the focus on the merits of consuming whole grain foods is important.

Thermal Processing

Processing of food using heat is the most common method of food processing and preservation. Apart from the preservation effect, thermal processing can influence other quality aspects such as sensory, nutritive and phytochemical quality. Heat processing can take up various forms including cooking, roasting, microwave heating and extrusion cooking, to mention a few. The severity and mode of the thermal process also has a bearing on the quality of the food. The literature seems to show that thermal processing of grains may increase or decrease phenolic content and antioxidant activity. Various mechanisms have been proposed to account for this including release of bound phenolics from the food matrix, polymerization and oxidation of phenolics, thermal degradation, depolymerization of high molecular weight phenolics such as condensed tannins and production of Maillard reaction products.

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Table I. Effects of various processing methods on phenolics and antioxidant activity of cereals and legumes

Cereal / Legume	*Results*	*Reference*
Decortication / Dehulling		
Sorghum	Reduction in total phenolics by 70-85% due to decortication to 70% extraction rate	(*21*)
Wheat	Increasing degree of pearling reduced the antioxidant capacity of the pearled grains and their by-products. Unprocessed wheat grains had higher antioxidant capacity than the pearled grains.	(*22*)
Barley	General progressive decrease in total phenolics, antioxidant capacity and levels of phenolic acids (vanillic, caffeic, p-coumaric, ferulic and sinapic acids) with increasing degree of pearling.	(*23*)
Sorghum	Decortication to extraction rates of 70-81% reduced total phenolics by 33-77% and antioxidant activity by 73-87%.	(*24*)
Sorghum	Decortication to 70% extraction rate reduced total phenolic content by 43-66% and antioxidant activity by up to 10 times.	(*25*)
Finger millet	Progressive decrease in total phenolic content with increasing removal of bran fraction.	(*26*)
Pearl millet	Dehulling reduced total polyphenol content by approximately 50%.	(*27*)
Thermal processing		
Wheat, barley, rye and oat	Extrusion cooking produced increases in free and ester-bound phenolic acids by 200 to 300%.	(*28*)
Sorghum	Cooking (boiling the meal) reduced proanthocyanidin levels by 54%.	(*29*)
Sorghum	Extrusion cooking reduced total phenolics, tannin contents and antioxidant activity of whole and decorticated tannin sorghums	(*24*)
Navy bean and pinto bean	Pre-dehulling heating treatments (100°C) increased total phenolics and antioxidant activity significantly in seed coats and whole seeds	(*30*)

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Table I. (Continued). Effects of various processing methods on phenolics and antioxidant activity of cereals and legumes

Thermal processing		
Sprouts and seedlings of wheat, buckwheat, corn and oats	Thermal processing by autoclaving generally led to higher total phenolic content and DPPH radical scavenging activity.	(*31*)
Cowpea, pea and kidney bean	Boiling, roasting, microwave heating, autoclaving, and micronization caused significant decreases in tannin content in all legumes studied. Highest reduction was caused by boiling followed by autoclaving and microwave heating.	(*32*)
Chickpea, lentil and bean	Cooking reduced total phenolic contents of the legumes by 21 to 50%. Cooking reduced proanthocyanidin content of the legumes and dehydration after cooking brought about further reductions.	(*33*)
Bean	Cooking (independent of cooking temperature) without soaking and without discarding the cooking water led to enhanced total phenolics and antioxidant activity and increase in flavonols and free phenolic acids.	(*34*)
Barley	Cooking (regular boiling under atmospheric pressure) and roasting (125°C for 30 min) significantly increased total phenolic content and DPPH radical scavenging activity, with roasting producing a higher increase. Cooked and roasted grains had similar capacity to inhibit LDL oxidation.	(*35*)
Durum wheat pasta enriched with debranning fractions of wheat	Pasta production using an extrusion process decreased the amount of phenolic acids in the free phenolic fraction but no change for the bound phenolic fraction. Cooking (by boiling) the pasta increased the phenolic acid content of the bound fraction while the free fraction generally did not vary. Cooking also increased ABTS radical scavenging capacity of the pasta.	(*36*)

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Table I. (Continued). Effects of various processing methods on phenolics and antioxidant activity of cereals and legumes

Thermal processing		
Little millet (Panicum sumatrense)	Steaming and roasting (165°C for 75 s) significantly increased total phenolics, total flavonoids and tannin content. The increase on roasting was higher than on steaming. The thermal treatments also increased DPPH radical scavenging activity and reducing power.	(*37*)
Cashew nut	Low temperature (70°C for 6 h) and high temperature (130°C for 33 min) roasting increased total phenolic content of souble and bound phenolic fractions of the whole cashew nut, the kernel and testa. Antioxidant activities were increased as roasting temperature increased.	(*38*)
Malting / Germination		
Sorghum	Progressive decrease in phenolic content with increase in germination time.	(*39*)
Sorghum	Malting of tannin sorghum grains decreased soluble proanthocyanidins by 48 and 59%. Total phenol contents of two tannin sorghums and a non-tannin sorghum were reduced during malting. Total phenol content of one other non-tannin sorghum increased marginally.	(*40*)
Beans, lentils and peas	Overall increase in hydroxybenzoic acids during germination. Some hydroxycinnamic acids that were not detected in raw seeds, were detected after germination. Some flavonol glycosides that were absent in raw beans were detected after germination. Procyanidins present in raw lentils were not detected after germination. Germination increased antioxidant activity for beans and peas but decreased for lentils.	(*41*)
Mung bean and soybean	Germination increased total phenolic content, peroxyl radical trapping capacity, ABTS radical scavenging capacity and inhibition of lipid peroxidation.	(*42*)

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Table I. (Continued). Effects of various processing methods on phenolics and antioxidant activity of cereals and legumes

Malting / Germination		
Various edible seed species	General increases in total phenolic content and DPPH antiradical activity after germination for 7 days. Increase in total phenolics ranged from 2010% (mungbean) to –11% (kale) and increases in antioxidant activity ranged from 1928% (mungbean) to 0% (lentil).	(*43*)
Barley	Significant decrease in total phenolic content after 12 h of germination, but a subsequent increase after 24 h germination period. Antioxidant activity increased after 12 h germination and increased further after 24 h.	(*44*)
Barley	Germination for about 48 h reduced total phenolic content.	(*35*)
Little millet (Panicum sumatrense)	Germination at 25°C for 48 h significantly increased total phenolics, total flavonoids, tannin content and reducing power.	(*37*)
Fermentation		
Sorghum	Traditional spontaneous lactic acid fermentation reduced proanthocyanidins by 54 and 63%.	(*40*)
Sorghum	Lactic acid and alcoholic fermentation reduced proanthocyanidin levels by 52 and 34% respectively. p-Hydroxybenzaldehyde levels decreased during both lactic acid and alcoholic fermentation. p-Hydroxybenzoic acid and p-hydroxybenzoyl alcohol levels increased during alcoholic fermentation.	(*29*)
Cowpea (Vigna sinensis)	Fermentation (naturally or with Lactobacillus plantarum) or autoclaving after fermentation decreased peroxyl radical trapping capacity but increased ability to inhibit lipid peroxidation and radical scavenging capacity.	(*45*)
Sorghum	Fermented slurries of whole and decorticated tannin sorghum grains and their fermented porridges had lower total phenolic content and antioxidant activity compared to unprocessed grains	(*24*)

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Table I. (Continued). Effects of various processing methods on phenolics and antioxidant activity of cereals and legumes

Fermentation		
Rice	Fermentation using a self-designed bioreactor and conventional solid-state fermentation both led to increases in total phenolic content and DPPH radical scavenging activity.	(*46*)
Cowpea, pea and kidney bean	Fermentation with Saccharomyces cerevisiae reduced tannin content in all legumes studied significantly.	(*32*)
Buckwheat, wheat, barley and rye	Fermentation with Lactobacillus rhamnosus A71 and Saccharomyces cerevisiae both increased total phenolic content and antioxidant activity	(*47*)
Black soybeans	Solid state fermentation with Bacillus subtilis BCRC 14715 led to increases in total phenolics, total flavonoids, DPPH radical scavenging activity and Fe^{2+} chelating ability.	(*48*)
Sorghum	Chemically acidified sorghum doughs had decreased levels of phenolic acid esters and increased concentration of all phenolic acids. There was a reduction in levels of flavonoid glucosides in the chemically acidified dough with corresponding increase in levels of aglycones. Fermentation with Lactobacillus strains likewise reduced levels of glycerol esters of phenolic acids. Levels of phenolic acids in the microbially fermented sorghum were significantly lower than in the chemically acidified dough probably due to their being metabolized during fermentation.	(*49*)
Nixtamalization		
White and blue corn types	Decrease in total phenolic content, anthocyanin content and antioxidant activity upon nixtamalization with further decreases when masa was processed into tortillas. However, nixtamalization produces the greatest decreases in phenolics and antioxidant activity. Lime cooking also increased free ferulic acid content of the grains.	(*50*)
Corn (white, yellow, high-carotenoid, blue and red types)	Nixtamalization decreased total phenolics and anthocyanin content, increased free and soluble-conjugated ferulic acid and decreased bound ferulic acid. There were	(*51*)

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Table I. (Continued). Effects of various processing methods on phenolics and antioxidant activity of cereals and legumes

Nixtamalization		
	increases in hydrophilic and lipophilic antioxidant activity of free phenolics but decreases in hydrophilic and lipophilic antioxidant activity of bound phenolics.	
Tortillas produced from nixtamalized and extruded flours from blue, red, white and yellow corn types	Both nixtamalization and extrusion cooking reduced total phenolics of tortillas with the reduction being more severe with the nixtamalization process. White corn tortillas from nixtamalized flour had the lowest retention of phenolic compounds. Levels of free ferulic acid in tortillas prepared with nixtamalized flours were higher compared to tortillas produced from extruded flours. Anthocyanin levels of blue corn tortillas were reduced by both nixtamalization and extrusion cooking.	(*52*)
Irradiation		
Rice hulls	Far infrared radiation treatment of rice hulls for 30 min increased total phenolic content, DPPH radical scavenging activity and ability to inhibit lipid peroxidation. Higher levels of various phenolic compounds were detected in the far infrared-treated rice hulls.	(*53*)
Velvet bean seeds	Dose-dependent increase in total phenolics (5-30 kGy) and tannins (10-30 kGy).	(*54*)
Pearl millet	Gamma irradiation (30 and 60 kGy) had no significant effect on total polyphenols in whole and dehulled pearl millet flour.	(*27*)
Pistachio hulls	Gamma irradiation (10 kGy) decreased total phenolic content but at 20-60 kGy, total phenolic content increased. Irradiation reduced tannin content and antioxidant activity.	(*55*)

Randhir et al. (2008) (*31*) investigated the effects of thermal processing in the form of autoclaving on total phenolics, antioxidant activity and other health-relevant functional properties of wheat, buckwheat, corn and oats sprouts and seedlings. They observed that thermal processing brought about softening and browning of plant tissues and increased total phenolic content and antioxidant activity. Similar increases in total phenolic content due to thermal processing by

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autoclaving have been reported in corn (*64*) and some legume (fenugreek, soybean, fava bean and mung bean) sprouts and seedlings (*65*). Randhir et al. (2008) (*31*) provided a number of reasons for the observed increases in total phenolic content and antioxidant activity upon thermal processing. In general, these mechanisms apply to general increases in phenolic content in thermally processed grains.

- Thermal processing of grains could bring about release of bound phenolics from the breakdown of cellular components and cell walls. This is also known as phenolic aglycosylation and could lead to increased antioxidant activity (*66*). In this regard, increase in antioxidant activity of thermally-processed sweet corn has been attributed to increase in solubilized ferulic acid esters and release of bound phenolics from the cell matrix (*67*).
- Further polymerization and oxidation of the phenolic constituents may also contribute to the observed increase in total phenolics and antioxidant activity (*68*).
- Other phenolics apart from those endogenously present in the grains could be formed as a result of thermal processing. For example, high molecular weight phenolics such as tannins may be thermally degraded to simple phenolics. Chen et al. (2006) (*69*) reported increases in phenolic acids such as ferulic, syringic, vanillic and p-coumaric acids in wheat flour upon thermal treatment.
- Phenolics that accumulate in cellular vacuoles (*70*) could also be released by thermal processing.
- Products of the Maillard reaction and caramelization may increase phenolic content on thermal processing (*25*, *71*).
- The structure of phenolic compounds has a bearing on their action as antioxidants (*72*). Thermal processing may change phenolic structure in such a way as to improve antioxidant function.
- There may be additive or synergistic effects between other phytochemicals and thermally altered phenolics.

Fares et al. (2010) (*36*) determined the effect of processing semolina into pasta and cooking on phenolic acids profile and antioxidant properties of pasta samples enriched with wheat debranning fractions and found both decreases and increases in phenolic content due to processing. Specifically, there was a decrease of free phenolic acids in control and wheat bran-enriched samples on processing semolina into pasta, mainly due to a decrease in p-hydroxybenzoic acid. There was however no change in the bound phenolic acid fraction. Cooking the pasta increased the levels of bound phenolic acids with generally no variation in free phenolic acid levels. This is somewhat in contrast with Zielinski et al. (2001) (*28*) who reported increases in free and bound phenolic acids after severe hydrothermal processing of some cereal grains.

According to Fares et al. (2010) (*36*), during pasta processing, oxygen, water and heat treatment induce oxidative degradation of antioxidants including phenolics as suggested by other authors (*73*). This may account for the observed decrease in free phenolics on pasta processing which are considered to be more

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reactive than bound phenolics in counteracting the effects of oxygen and heat. On the other hand, during cooking, boiling water can enhance the release of bound phenolics from the food matrix such as ferulic acid ester-linked to cell walls. This could increase bound phenolics content and antioxidant activity. Fares et al. (2010) (*36*) also mentioned the possibility of Maillard reaction products produced during the cooking process contributing to enhanced overall antioxidant activity (*71*).

Dlamini et al. (2007) (*24*) reported that extrusion cooking reduced total phenolics, tannin contents and antioxidant activity of whole and decorticated tannin sorghums. Other authors (*74*) also reported some reduction in antioxidant activity of sorghum upon extrusion cooking but not to the same extent as observed by Dlamini et al. (2007) (*24*). It appears that the extrusion conditions influence extent of retention or loss of phenolics and antioxidant activity. It is suggested (*24*) that higher moisture content (approx. 18%) used during the extrusion process could promote phenolic and tannin polymerization (*75*). On the other hand, lower moisture content (< 15%) coupled with high shear and high temperature during extrusion may depolymerize condensed tannins into more extractable lower molecular weight oligomers (*74*). Extrusion may also promote release of bound phenolics from components such as proteins and cell walls (*24*).

Malting or Germination

Malting, in simple terms, can be defined as the germination of grain in moist air under controlled conditions (*56*). Sorghum and barley are the two principal cereal grains that are malted and used in the production of opaque beer (sorghum malt) and lager beer (both sorghum and barley malt). Malt is also used to produce various non-alcoholic beverages and malt-flavored drinks, in breakfast cereals and baked goods and confectionery. Legume sprouts (e.g. bean sprouts) are produced through a process of germination and form an important part of diets particularly in countries of the Far East.

The malting process involves wetting (by steeping) and germination of the grain under carefully controlled conditions to bring about desirable physical and chemical changes associated with the germinative process. During malting, various endogenous enzymes of the grain are mobilized including amylases, proteases, lipases, cell wall degrading enzymes and phytases. The action of these enzymes brings about modifications in the chemical constituents of the grain such as starch, proteins, cell walls and subsequently, the structure of the grain. Phenolic compounds in grains are mostly found closely associated with these chemical constituents such as proteins and non-starch polysaccharides in cell walls (*7*). The influence of malting or germination on phenolic compounds is therefore based on the metabolic processes that occur during germination and the action of the endogenous enzymes on the chemical constituents of the grain. Most workers have reported an increase in phenolic content of grains during malting or germination, although decreases have also been reported in some cases.

Shibuya (1984) (*76*) reported that the increase in phenolic compounds in germinated brown rice could be due to the increase in the free phenolics forms due to dismantling of the cell wall during germination. Tian et al. (2004) (*77*)

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reported that during germination of rice the hydrolytic action of amylase enzymes on starch led to release of bound phenolic compounds with increasing total phenolic content as a result. There was also a significant increase in free phenolic acid content during germination. Ferulic acid content of brown rice also increased and became the most abundant phenolic compound in germinated brown rice.

Fernandez-Orozco et al. (2008) (*42*) reported significant increase in total phenolic content of mung bean after up to 7 days of germination and of soybean after up to 6 days of germination. Germination also increased antioxidant activity in terms of peroxyl radical trapping capacity, antioxidant activity in a liposomal phosphatidyl choline system and ABTS radical scavenging. Germination has also been reported to increase total phenolic content (*78*) and flavonoid content (*79*) of soybeans. Zielinski (2002) (*80*) reported increase in peroxyl radical scavenging capacity of soybean seeds after germination. Germination of legumes increases inhibition of phosphatidyl choline liposome peroxidation (*81*, *82*). Changes in peroxyl radical trapping capacity during germination also appears to be dependent on the type of seed (*81*, *83*).

Sharma and Gujral (2010) (*44*) reported a decrease in total phenolic content of barley after 12 h of germination which increased after germination for 24 h. Antioxidant activity however increased after both 12 and 24 h of germination. These authors suggested that the decrease in total phenolic content after germination for 12 h may be due to the phenolic compounds being metabolized to other compounds and their leaching into the steeping water (*84*). Phenolic compounds may also form of insoluble complexes with proteins that hinder their extraction (*85*). On the other hand, the increase in total phenolic content after germination for 24 h may be due to bound phenolic compounds becoming free by the action of enhanced hydrolytic enzyme activity (*86*).

Fermentation

Fermentation refers to the process whereby the activity of microorganisms (usually anaerobic) on suitable substrates under controlled or uncontrolled conditions results in the production of foods or beverages that are usually more stable, palatable and nutritious than the raw substrate (*87*). Fermentation is considered as one of the oldest methods of food processing and preservation. Worldwide, a wide range of fermented foods and beverages play an important role and contribute significantly to the diets of many people.

Seven principal fermentation reactions in food are listed as follows: lactic acid fermentation, propionic acid fermentation, citric acid fermentation, alcoholic fermentation, butyric acid fermentation, gassy fermentation and acetic acid fermentation (*87*). Various genera of bacteria (Acetobacter, Streptococcus, Leuconostoc, Pediococcus, Lactobacillus and Propionobacterium), yeast (Saccharomyces, Candida and Torula) and mold (Aspergillus, Penicillium, Rhizopus and Mucor) are used in food fermentation. The metabolic activities of these microorganisms which also involves a variety of enzyme activities have a significant effect on the chemical constituents of the food including phenolic compounds and their bioactive properties. Such effects may include binding of

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phenolics to other plant constituents such as proteins which makes these phenolics unextractable or degradation of phenolics by microbial enzymes.

Fermented slurries of whole and decorticated tannin sorghum grains and their fermented porridges had lower total phenolic content and in vitro radical scavenging activities compared to unprocessed grains (*24*). It was suggested that these observations may be due to changes during fermentation that affect extraction of total phenols and tannins; may involve associations between tannins, phenols, proteins and other compounds in the grain. Similar reductions in tannin content of sorghum due to fermentation have been reported (*88*, *89*). It has been suggested that tannins can bind with proteins and other components in aqueous media as encountered during fermentation (*90*). This will then reduce their extractability. Tannins may also be degraded by microbial enzymes during fermentation (*91*). For instance, polyphenol oxidase has been reported to reduce phenolic content in tannin sorghums.

A progressive decrease in total phenolic content of two pearl millet cultivars with increasing fermentation time has been reported (*62*). The reduction was by up to 2.5 times after 14 hours of fermentation and it was suggested that this could be due to microbial activity during the fermentation process. On the other hand, Doblado et al. (2005) (*45*) observed that fermentation increased antioxidant activity in a liposomal phosphatidyl choline system and ABTS radical scavenging capacity of Vigna sinensis. This study showed that the fermentation of cowpeas (naturally or with L. plantarum) and subsequent heat treatment in an autoclave were good processes to obtain functional cowpea flours having higher antioxidant capacity than the raw legume.

Nixtamalization

Corn tortillas are a staple food of Mexico and Central America and have gained popularity as a salted snack food in the world. Their production involves a thermal alkaline treatment or nixtamalization process (*92*). During nixtamalization, the corn kernels are cooked in a lime or calcium hydroxide solution and steeped overnight. The resulting nixtamal is then washed and ground into dough (masa) which is further processed into tortillas (*92*). The nixtamalization process has profound effects on some physico-chemical, nutritional and sensory properties of the corn kernels and resultantly, the food products made from them. Nixtamalisation can remove the pericarp of the corn kernels (*92*), incorporate calcium into the kernels (*93*), improve bioavailability of niacin (*94*) and also lead to formation of flavor and color compounds that are important for sensory quality (*92*).

As would be expected, nixtamalisation does bring about losses in phenolic compounds and antioxidant activity of corn and food products from the nixtamalised corn. The alkaline treatment hydrolyzes fiber components located in the pericarp, aleurone and endosperm cell walls such as hemicelluloses and lignin to which phenolics are bound (*51*, *95*). These phenolics are thus released, solubilized and they subsequently leach into the alkaline steep liquor which is also known as nejayote. Infact the nejayote is considered to be even richer in phenolics than the original corn kernels and masa (*96*). Considering the process

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of producing the corn tortillas as a whole, the nixtamalisation process appears to bring about the most significant losses in phenolic antioxidants (*50*).

The degradation of anthocyanins is also an important effect of nixtamalization (*50–52*, *97*, *98*). Alkaline conditions prevailing during nixtamalization adversely affect stability of anthocyanins and lead to losses (*99*, *100*). It is hypothesized that the alkaline pH conditions prevailing during nixtamalization (approximately pH 10) and the temperature exert synergistic effects and bring about structural changes and reduce stability of anthocyanins (*51*).

Irradiation

Irradiation processing of food is a well established physical, non-thermal method of food processing. The attractiveness of this technique lies in the fact that it causes minimal modification of flavor, color, nutrients, taste and other quality attributes of food. The extent of food quality by irradiation depends on factors such as the raw material used and the type of radiation source used (gamma, X-ray, UV, electron beam) (*54*, *101–103*).

Short wavelength and high frequency forms of radiation such as gamma rays and X-rays are sufficiently high enough in energy to cause ionizing. The ions produced decompose rapidly to form highly reactive free radical species. The observed chemical and biological effects of radiation are due to the action of these free radical species. As a result, in any food matrix consisting of various constituents such as proteins, carbohydrates, fats, vitamins and water, a lot of complex chemical reactions can occur (*104*). These events during food irradiation will therefore have a bearing on phenolic compounds and antioxidant activity of the food.

Alothman et al. (2009) (*101*) have authored an excellent review on the effects of radiation processing on phytochemicals and antioxidants in plant produce. Irradiation may lead to increases or decreases in the levels of phenolic phytochemicals in food. Various factors such as the irradiation dose applied, sensitivity of the phenolic compound to irradiation and the effect of the irradiation itself on other food constituents play a role in determining the overall effect of irradiation processing on phenolics in food (*101*).

Levels of luteolin in methanolic extracts of uv-irradiated peanut hulls were reduced by up to 69.5% after irradiation for 6 days while concentration of total phenolic compounds were reduced by up to 90.4% (*105*). Irradiated samples still showed high antioxidant activity. El-Niely (2007) (*106*) reported reduction in total phenolic content of various legumes on gamma-irradiation up to a dose of 10 kGy. Gamma-irradiation (2 kGy) or cooking on their own had no significant effect on total phenolics in pearl millet flour (*27*). However, irradiation in combination with cooking reduced total phenolics. Toledo et al. (2007) (*107*) also reported reduction in total phenolics of soybean grains on irradiation and cooking.

Increases in phenolic content on irradiation have also been reported by various authors and this is attributed for the most part, to release of phenolics from the food constituents to which they are bound such as cell walls as a result of the irradiation process. Variyar et al. (2004) (*108*) reported a significant decrease in total isoflavones and glycosides in soybean with increased irradiation

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dose (0.5-5.0 kGy), however the aglycone content showed an increasing trend. Also, a significant increase in the percentage of DPPH scavenging activity with increased gamma-irradiation was observed probably related to the increased aglycone content. It was suggested that irradiation can induce breakdown of glycosides resulting in release of free isoflavones (*108*). Harrison and Were (2007) (*109*) also observed a significant increase in total phenolics and antioxidant activity of almond skin extracts after gamma irradiation (0-16 kGy) which they attributed to the release of phenolic compounds from glycosidic components and the degradation of larger phenolic compounds into smaller ones by gamma irradiation.

Bhat et al. (2007) (*54*) determined the effect of gamma irradiation on antinutritional components of seeds of Mucuna pruriens (a wild legume in tropical and sub-tropical regions of the world) on exposing to doses of 2.5, 5.0, 7.5, 10, 15 and 30 kGy. Except for 2.5 kGy, the rest showed significant dose-dependent increase in phenolics. Tannin concentration did not differ significantly up to 7.5 kGy, while it significantly increased at higher doses. They attributed such increase in phenolics to higher extractability by depolymerization and dissolution of cell wall polysaccharides by irradiation as has been reported by Siddhuraju et al. (2002) (*110*) who observed increased phenolics in green gram (Vigna radiata) seeds on soaking, followed by irradiation. Increase in phenolics on irradiation may also be attributed to irradiation-induced increase in activity of the enzyme phenylalanine ammonia-lyase which is responsible for synthesis of phenolic compounds (*111*).

Concluding Remarks

It is clear that the effects of processing on phenolic compounds and antioxidant properties are varied and wide ranging. Various mechanisms are at play in determining the fate of phenolics during processing. The observation that phenolics in the food matrix can be released and therefore made potentially available for absorption underscores the need for careful control of processing conditions to enhance levels of phenolics and minimize their degradation. Although there is a wealth of information currently available, there is still a need for more research into how bioactive compounds in cereal and legume grains are affected by processing. The methods for measuring bioactive properties of processed cereal and legume foods are essentially in vitro in nature. It will be a significant step forward for this field of research if these in vitro effects can be extended to effects in vivo in order to directly link phenolic compounds and other bioactives to specific health effects with more accuracy and surety.

Dedication

This chapter is dedicated to the memory of my mother, Lucy Abena Oguamena Osei, who passed away during the preparation of this manuscript. May her soul rest in peace.

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Chapter 4

Metabolite Profiling of Cereals – A Promising Tool for the Assessment of Grain Quality and Safety

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Metabolite profiling represents an approach for the simultaneous detection, identification and quantification of a broad spectrum of low molecular weight metabolites in complex biological systems. The unbiased and non-targeted screening of metabolic profiles in combination with appropriate multivariate and univariate statistical tools enables the evaluation of genetics and environment-related impact factors on cereal grain quality and safety. In this context, the assessment of the extent of variation in the light of natural variability constitutes a major challenge. The application of metabolite profiling may also increase the probability to detect effects not intended by genetic modifications, e.g. through genetic engineering or mutation breeding. Untargeted metabolite profiling-based investigations of (i) genetically modified maize, (ii) rice mutants, and (iii) barley in the course of the malting process are presented demonstrating their suitability for the assessment of cereal grain quality and safety.

Introduction

Unbiased profiling techniques, so called "omics"-techniques, are considered as powerful tools in the field of functional genomics such as DNA sequencing, transcription profiling, protein and metabolite analyses (*1*). Ultimate objectives are to reveal comprehensive information on the genome (genomics), transcriptome

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(transcriptomics), proteome (proteomics) and metabolome (metabolomics) in biological systems. Metabolites represent the end products of the interactions between the genome, transcriptome and proteome with the environment and thus define the biochemical phenotype of a system (*2*, *3*).

Metabolomics-based profiling methods have been shown to provide valuable information in various fields of plant / food analysis. Such methods can result in important data for breeding-driven metabolic engineering of nutritionally important metabolites in crops (*4–6*) and may enhance the power of functional genomics (*7*). Functional genomic approaches have been applied in the analysis of quality traits of major cereals for further improvement and the development of new cultivars (*8*). Comprehensive datasets on metabolites contribute to the understanding of crop metabolic interactions and may thus also help to improve agronomic characteristics (*9*, *10*). During the past decade, metabolomics techniques have been applied to the assessment of phenotypic diversity in plants (*11*), to comparative investigations of breeding systems, e.g. conventional vs. genetically modified crops (*12*) and farming practices (*13*) and to the determination of environmental impacts on the variability of crop metabolites (*14*).

Before introducing genetically modified crops to the market, comprehensive safety assessments are required. On the crop composition level, targeted analytical methods are used for the analysis of specific key compounds. However, due to their unbiased character, metabolomics-based profiling methods are also being discussed as additional tools for the safety assessment of genetically modified crops as they may increase the probability to detect unintended effects (*15*, *16*).

This chapter demonstrates the potential of metabolomics-based analyses for the assessment of quality and safety of cereal grains. The application of commonly used metabolomics-based techniques in combination with an appropriate statistical data analysis workflow is shown for cereal grain genotypic and phenotypic investigations. Case studies related to genetic engineering, mutation breeding and processing of cereal grains are presented demonstrating the suitability of the employed untargeted approach (i) to assess the influence of genetic modification on grain metabolic profiles in the light of natural variability, (ii) to assist in the elucidation of mutation events and (iii) to follow processing-related changes in cereal metabolites profiles and thus to contribute to an adequate nutritional quality and safety of the final food.

Metabolite Profiling

Techniques

Two major types of approaches are being applied to perform metabolomics-based investigations. Metabolic fingerprinting represents a rapid screening method for biological samples without a major pre-treatment of the material to be analyzed. In order to screen a large number of these samples, e.g. in plant breeding programs, it is not always envisaged to determine the individual

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levels of each metabolite. Instead, a rapid classification of samples might be sufficient. Methods like nuclear magnetic resonance (NMR), Fourier transform infrared (FT-IR) spectroscopy and mass spectrometry (MS) are used within this approach (*2*). Metabolite profiling represents the analysis of selected compounds from the same chemical compound classes or compounds linked by known metabolic relationships. Metabolite profiling can be considered as one of the most pragmatic approaches presently applied. It aims at the detection, identification and quantification of a broad spectrum of compounds in a single sample in an effective and reproducible way to provide a deeper insight into the complex biological system (*2*, *17*, *18*). For the analysis of metabolites, different technology platforms have been established. Coupling of capillary gas or liquid chromatography and mass spectrometry (LC/MS, GC/MS) have proven to be the best understood and most applied methodologies within the scope of plant metabolomics investigations (*19–23*).

Workflow

The application of metabolite profiling to broad arrays of samples results in a huge amounts of metabolite-related data. Therefore, the traditional data analysis based on "value-by-value" comparisons is not satisfactory. For metabolite profiling data it is more appropriate to start with a multivariate analysis approach for the overall determination of variation in a dataset followed by univariate analysis of metabolites shown to be relevant in the foregoing multivariate approach.

Multivariate statistical analytical methods such as principal component analysis (PCA), hierarchical cluster analysis (HCA) and self-organizing maps (SOMs) proved to be useful tools in the analysis and evaluation of metabolite profiling data (*3*, *12*, *24*). Principal component analysis enables the rapid differentiation of samples based on their metabolite profiles by visualizing the data as dots in a two-dimensional plot (Figure 1). The investigation of the major sources of variation is followed by a substantiation of differences. An efficient way to identify the drivers of variation is to examine the factor loadings as determined by PCA. For example, if a clear separation of samples is revealed on the first principal component, the main contributors to this effect will exhibit high absolute loading factors in PC1 (Figure 1). In a subsequent step, the signals of target compounds can be evaluated using univariate statistical approaches such as Student´s t-test and analysis of variance (ANOVA). A good visualization of univariate data is obtained by the use of box plots where the data can easily be compared to data sets representing natural ranges (Figure 1).

A major future challenge for metabolomics will be the implementation of reporting standards as suggested by the Metabolomics Standards Initiative (*25*). This would ensure the comparability of metabolite profiling data to be used for crop metabolite databases.

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Safety and Quality Assessment

Genetic Engineering

Genetic engineering of agricultural crops is being employed for yield improvement, e.g. by increasing resistance to disease (*26*) and stress (*27*) and tolerance to herbicides (*28*), as well as for improvement of the nutritive value of crops, e.g. by increasing the availability of essential micronutrients (*29*, *30*). However, before introducing genetically modified (GM) crops to the market, comprehensive safety assessments are required. A key element in the safety assessment of GM crops, originally introduced in the beginning of the 1990s by the Organisation of Economic Co-operation and Development (OECD) (*31*), is the concept of "substantial equivalence".

It is based on the idea, that if a GM crop is found to be substantially equivalent to an existing food, it can be treated in the same manner with respect to safety as the commonly accepted conventional comparator. Safety assessment procedures for GM foods have been extensively reviewed in literature (*16*, *32–34*). The concept is used to identify similarities and differences between the GM crop and a conventional comparator and includes a comprehensive characterization, e.g. molecular analysis, assessment of agronomic performance and investigations of the chemical composition. For GM-derived crops, near isogenic lines are usually employed as conventional comparators within a safety assessment. Expected genotypic and phenotypic alterations as a result of genetic engineering can be determined by targeted analytical approaches. In addition, on the food composition level, targeted compositional analyses have been suggested for the assessment of specific safety and nutrition-related compounds, ranging from macronutrients to anti-nutrients and naturally occurring toxicants (*31*, *35*). On this basis, numerous targeted compositional investigations of genetically modified cereals have been published demonstrating substantial equivalence to the respective conventional comparator (*36–41*).

A challenging goal is the detection of effects not intended by genetic modification (*16*, *32*, *42*). Targeted analytical approaches for the detection of potential unintended effects may have limitations owing to their biased character. Therefore, unbiased non-targeted metabolite profiling approaches are being discussed as additional tools for the safety assessment especially of genetically modified crops. Their potential role has been extensively reviewed (*15*, *16*, *43–45*). Comprehensive overviews on metabolite profiling-based comparative studies of genetically modified and non-GM crops including barley, rice, wheat and maize are available (*46*, *47*).

Bt Maize and Roundup Ready Maize

Maize as one of the most important agricultural crops and as part of the staple diet of humans and livestock has been subjected to a variety of genetic modifications. Transgenic maize plants have been produced with different characteristics including insect-resistant *Bacillus thuringiensis* (Bt)-maize and herbicide-tolerant Roundup Ready maize. In the present case study, two sets of

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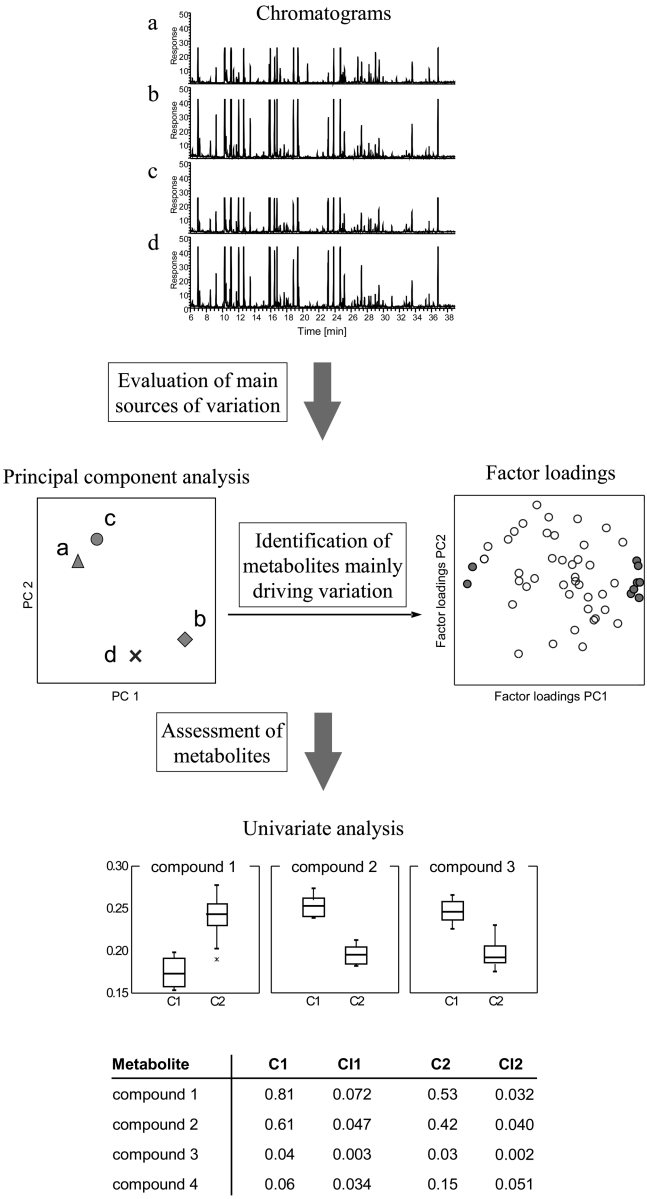
transgenic maize lines (Bt- and Roundup Ready) and their isogenic counterparts grown in South Africa and Bavaria (Germany) were analyzed by capillary gas chromatography mass spectrometry metabolite profiling.



Metabolite	C1	CI1	C2	CI2
compound 1	0.81	0.072	0.53	0.032
compound 2	0.61	0.047	0.42	0.040
compound 3	0.04	0.003	0.03	0.002
compound 4	0.06	0.034	0.15	0.051

Figure 1. Typical workflow of capillary gas chromatography-based metabolite profiling.

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To assess the influence of genetic modification under different environmental conditions, Bt-maize was grown together with its near isogenic line (non-GM) at three locations in South Africa (Petit, Lichtenburg, Potchefstroom) in one growing season. In addition, at Petit and Lichtenburg Roundup ready-maize (GM-RR) was grown together with the Bt maize and the isogenic line. At Petit and Lichtenburg, the maize was grown applying high-input regimes, at Potchefstroom a low-input farming practice was conducted. Samples collected from the three growing locations were subjected to GC/MS-based metabolite profiling followed by multivariate data analysis, i.e. principal component analysis (Figure 2).

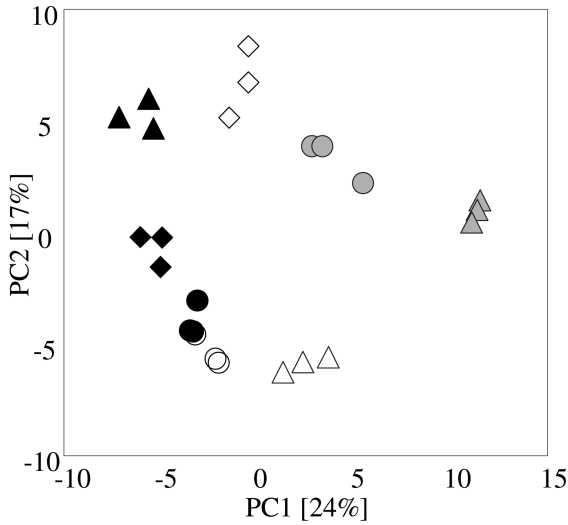

Figure 2. Principal component analyses of metabolite profiling data of Bt maize (shaded triangle, open triangle, solid triangle), Roundup Ready maize (open diamond, closed diamond) and the isogenic counterpart (shaded circle, open circle, solid circle) grown at the three locations Potchefstroom (gray symbols), Lichtenburg (white symbols) and Petit (black symbols) in 2004. Samples were analyzed in triplicate.

The first two principal components accounting for 41% of the total metabolic variation revealed a visual clustering of GM and isogenic maize lines for each of the three growing locations. However, compared to the clustering between GM and non-GM maize at each single location, the variation according to the different growing locations along PC1 is far more pronounced indicating a strong environmental impact on the maize metabolite profiles. For the determination of significant metabolic differences between GM and non-GM maize, a univariate "peak-by-peak" analysis was performed. For the three single growing locations, on average, 25% of the total compounds included for comparison (on average more than 100 compounds) were significantly different ($p < 0.05$) between Bt maize vs.

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isogenic line and RR maize vs. isogenic line, respectively. However, none of those compounds turned out to be consistently significantly different between the two GM lines and the isogenic counterpart at all three field trials.

Maize Grown in Germany

GM-Bt and non-GM maize were grown at two different farming locations in Bavaria, Germany, in the season 2004. Field replicates were collected and subjected to GC/MS metabolite profiling. Principal component analysis of the metabolite profiling data revealed a clear separation of the two farming locations on the first principal component representing 34% of the total variation in the dataset (Figure 3). Clusters according to genotypes could be observed within farming locations only on PC2 (10% of total variation).

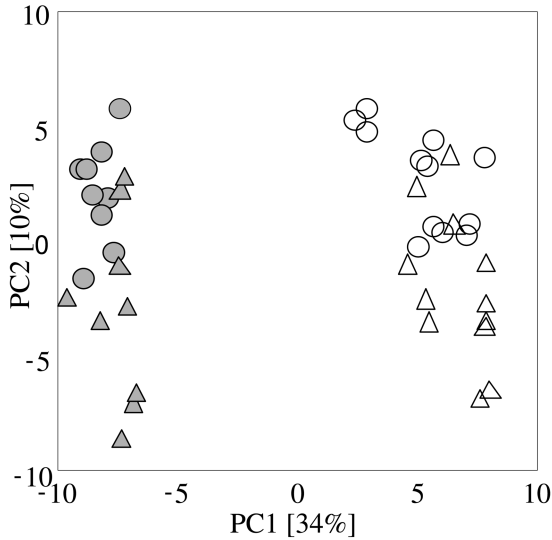

Figure 3. Principal component analysis of metabolite profiling data of Bt maize (shaded triangle, open triangle) and its isogenic counterpart (shaded circle, open circle) grown at Pfaffenhofen (shaded triangle, shaded circle) and Neuhof (open triangle, open circle). Three and four field replicates at Pfaffenhofen and Neuhof, respectively, were analyzed in triplicate.

Univariate data analysis revealed 12% and 19%, respectively, of the total covered peaks / compounds (on average 137 for the two growing locations) to be significantly different between Bt-maize and isogenic line. However, although only two field trials were analyzed, no consistent differences were observed.

The results obtained for the comparative metabolite profiling of the South African and the German GM maize and their respective isogenic counterparts are in agreement with data obtained for wheat showing that differences observed

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between GM and the control lines were generally within the same range as the differences observed between the control lines grown on different sites and in different years (*40*).

Impact of Genetic Engineering versus Natural Variability

On the level of chemical composition, a comparative analysis of the GM line and the respective non-GM line having a comparable genetic background forms the basis for the safety assessment of genetically modified crops. However, such a comparative analysis should not only focus on the GM crop itself and its corresponding parental line, but metabolite profiles should also be assessed in the light of natural variability that is inherent in conventional bred crops (*48*, *49*). Genetic background, growing environment (geographical, seasonal) and crop management practices are major factors underpinning this variation (*50*). Targeted studies with emphasis on maize grains demonstrated the impact of factors such as developmental stage (*51*), environment and farming practice (*52*, *53*) and genetic background and growing seasons (*36*, *54*, *55*) on the natural variability of metabolites.

In addition to targeted studies, non-targeted metabolite profiling has been applied to investigate the impact of genetics and environment (*56*), genetic modification (*57*) and farming practices (*13*) on maize grain metabolite profiles. As example, for the investigation of the impact of genetic background, growing location and season on maize grain metabolites, four maize cultivars, differing in their maturity classification and grown at four locations over three consecutive seasons, were subjected to a capillary gas chromatography-based metabolite profiling procedure (*14*). Multivariate data assessment by means of principal component analyses enabled the distinct clustering according to both maize cultivar and growing environment. Results obtained from the univariate analysis of the metabolite profiling data are shown in Table 1. On average, 20% of the total covered peaks were significantly different between the four maize cultivars grown at one location over three consecutive years. Compared to the genetic background, an even more pronounced impact was determined (on average 25% significant differences) considering the various maize cultivars grown in different seasons. The evaluation of natural variability due to farming locations revealed 13% significantly differences over the three growing seasons. Comparison of growing seasons within locations resulted in 14% significant differences; this is in the same range as observed for differences between farming locations.

The influence of genetic modification on maize metabolite profiles was assessed under different environmental conditions. The data generated revealed that environmental influences had occasionally stronger overall effects on the metabolome of the investigated maize genotypes than the genetic modification. Similar results were also reported by a research program launched by the UK Food Standards Agency in order to assess the potential use of omics approaches in comparative analysis and their relevance to risk assessment procedures for the next generation of GM foods. Publications arising from these projects observed that the differences between conventional varieties were always significantly greater

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than the differences between the wild-types and their respective transgenics (*58*, *59*). Nevertheless, the data do not allow a general conclusion on the potential for unintended effects in GM crops and a case-by-case approval remains pragmatic. Indeed, for the GM maize investigated in the presented case study, the differences in the metabolite profiles of genotypes grown in different environments were significantly greater than the effects of the transgene.

Table 1. Pair-wise comparisons of the number of statistically significant differences between maize cultivars (*n*=4), growing locations (*n* = 4) and growing seasons (*n* = 3)

	Cultivars			*Locations*		
Growing season	*total*[a]	*diff.*[b]	*%*	*total*[a]	*diff.*[b]	*%*
2004	107	22	20	113	19	17
2005	108	16	15	107	16	15
2006	112	29	25	111	6	5
Total	109	22	20	110	14	13
2004 vs. 2005	111	24	22	110	17	15
2004 vs. 2006	115	43	37	113	16	14
2005 vs. 2006	111	17	16	111	12	11
Total	112	28	25	111	15	14

[a] Average peak number for pair-wise comparisons [b] Number of significantly different peaks ($p < 0.05$)

Mutation Breeding

Mutation breeding has a long history since the first induced mutation experiments on barley in 1928 (*60*). Meanwhile, a total of about 3000 mutant crop varieties had been developed (*61*).

In contrast to genetic engineering, mutation breeding is considered as a conventional breeding method with the consequence that for cereals obtained by mutation breeding no formal safety assessment is required. However, the occurrence of unintended effects is not a phenomenon specific to genetic engineering. This is particularly true for crop mutants of which the nature of the mutations underlying the exhibited phenotype is not known. Within the functional genomics approach, metabolite profiling may therefore be used as additional tool for the investigation and characterization of unknown mutation events and may help to assist in the elucidation of such events for the discovery of the gene underlying the mutant phenotype. In case of new plant varieties developed with traditional techniques, application of metabolite profiling for the assessment of the safety of these crops has been suggested (*15*, *32*).

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Low Phytic Acid (*lpa*) Rice Mutants

Phytic acid (*myo*-inositol 1,2,3,4,5,6-hexakisphosphate) represents the major storage form of phosphorus in mature seeds (*62*). The contents in cereals vary from 0.6 to 2.2% (*63*). Phytic acid is considered as an anti-nutrient as it limits the bioavailability of minerals such as iron, zinc and calcium by formation of indigestible chelates (*64*, *65*). In addition, phytic acid is poorly degraded in the digestive system of humans and non-ruminants (*66*).

During the past years, various efforts have been made to breed crops with lowered phytic acid contents. Genetic engineering has been successfully applied to produce low phytic acid (*lpa*) rice and maize (*67–69*). Alternatively to the production of transgenic plants, mutation breeding has been used to develop crops with lowered phytic acid contents. γ-Irradiation (*70*, *71*) and chemically induced mutagenesis (*72*, *73*) have been applied to generate various *lpa* cereals including maize (*72–74*), barley (*75*, *76*), rice (*77*, *78*) and wheat (*79*). These *lpa* mutants exhibit significantly reduced phytic acid contents ranging from -50% to more than -95% compared to wild-types. For the following case study, the two *lpa* rice mutants *Os-lpa*-XS110-1 and *Os-lpa*-XS110-2, generated by γ-irradiation from the wild-type Xiushui 110 and grown side-by-side at a total of nine field trials at different locations and seasons in China were subjected to a comparative metabolite profiling investigation (*80*). Phytic acid analysis revealed differently pronounced reductions in phytic acid contents for *Os-lpa*-XS110-1 (-46%) and *Os-lpa*-XS110-2 (-22%) compared to the wild-type. However, the nature of the mutations underlying the exhibited phenotypes was originally unclear.

Comparative Analysis of lpa Mutants and Wild-Type

Results obtained by comparative metabolite profiling of the mutant lines and the wild-type Xiushui 110 grown in different field trials are shown in Table 2.

For comparison of the wild-type Xiushui 110 and the *lpa* mutant lines *Os-lpa*-XS110-1 and *Os-lpa*-XS110-2, on average 122 and 113 peaks, respectively, were included for comparison. For each field trial on average 38% and 34% of the peaks included for comparison were significantly different between the wild-type and the two mutants. However, only a few of these significant differences turned out to be consistently present at all investigated field trials (n=9). Identification of these consistent differences revealed increased contents of phosphate in both mutant lines. Levels of *myo*-inositol, galactose and raffinose were consistently increased in *Os-lpa*-XS110-1, whereas the level of *myo*-inositol was decreased in *Os-lpa*-XS110-2 (Table 2).

Elucidation of Mutation Events

The first step in the biosynthesis of phytic acid is the conversion of D-glucose 6-phosphate to Ins(3)P_1 catalyzed by MIPS followed by phosphorylation steps of *myo*-inositol monophosphate to phytic acid (Figure 4).

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Table 2. Peak-based comparison of chromatograms obtained by metabolite profiling of wild-type rice Xiushui110 (XS110) and low phytic acid mutant lines *Os-lpa*-XS110-1 (*lpa*-110-1) and *Os-lpa*-XS110-2 (*lpa*-110-2)

	XS110 vs. lpa-110-1			*XS110 vs. lpa-110-2*		
Field trial, year	*total*[a]	*diff.*[b]	*%*	*total*[a]	*diff.*[b]	*%*
Hainan, 2003/04	123	38	31	118	27	23
Jiaxing, 2003/04	119	38	32	116	63	54
Hangzhou 1, 2003/04	115	45	39	113	35	31
Hangzhou 2, 2003/04	108	38	35	108	67	62
Hainan, 2005/06	144	58	40	128	32	25
Jiaxing, 2005/06	121	47	39	107	21	20
Hangzhou 1, 2005/06	118	50	42	107	28	26
Fuzhou, 2005/06	135	51	38	121	44	36
Guangzhou, 2005/06	111	48	43	101	24	24
mean	122	46	38	113	38	34
consistent differences[c]		4	3		2	2

[a] Number of total peaks (polar and non-polar metabolites) for comparison [b] Number of peaks significantly different between mutant line and wild-type ($p < 0.05$) [c] Number of peaks significantly different between mutant line and wild-type ($p < 0.05$) at all nine analyzed field trials

In addition, free *myo*-inositol formed through dephosphorylation of $Ins(3)P_1$ by *myo*-inositol monophosphatase (MIP) acts as intermediate in the biosynthesis of phytic acid. As shown in Figure 4, the biosynthesis of raffinose is linked to the early biosynthetic pathway of phytic acid. Raffinose is synthesized by attaching galactose to sucrose involving *myo*-inositol as a carrier of galactose activated as galactinol.

The metabolites shown to be significantly and consistently different between the wild-type and the *lpa* rice mutants, were found to be closely related to the biogenetic pathways leading to phytic acid (Figure 4). Consideration of these metabolic changes in the light of the routes involved in the biosynthesis of phytic acid indicated a disturbance in the early biosynthetic pathway of phytic acid in *Os-lpa*-XS110-2 and a mutation event affecting phosphorylation of *myo*-inositol in *Os-lpa*-XS110-1. In fact, the metabolite profiling-based prediction for *Os-lpa*-XS110-1 was in accordance with molecular mapping results, which placed the *lpa* mutation on a site very close to the locus that encodes the putative *myo*-inositol kinase (MIK) gene in rice (*77*).

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The numbers of statistically significant differences in metabolites between wild-type rice and the mutants at each field trial (on average 38% for *Os-lpa*-XS110-1 and 34% for *Os-lpa*-XS110-2) are in the same orders of magnitude as those determined for low phytic acid maize mutants. Application of a GC/MS metabolite profiling approach similar to the one employed in the present study revealed 10% and 29%, respectively, of the detected metabolites to be statistically significantly different between wild-type maize two different *lpa* maize mutants (*81*).

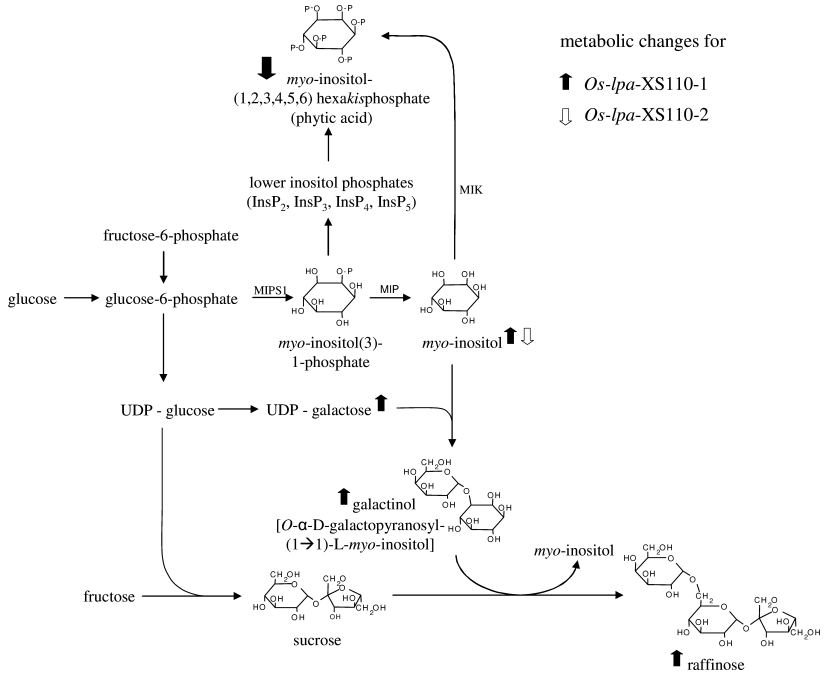

Figure 4. Link between biosynthetic pathways leading to phytic acid and raffinose and metabolic changes observed for the rice mutants Os-lpa-XS110-1 and Os-lpa-XS110-2 compared to the wild-type Xiushui110. MIPS1: 1D-myo-inositol 3-phosphate synthase; MIP: myo-inositol monophosphatase; MIK: myo-inositol kinase.

The assessment of statistically significant differences observed between wild-type rice and *lpa* mutants for consistency revealed that the vast majority of differences is related to biological variability rather than to the mutation event and that only a few metabolites remained as consistently different at all field trials. In order to assess the observed consistently significantly differences between the rice wild-types and low phytic acid mutants in the light of the natural variability, a conventional rice metabolite "model"-profiling sample database was created

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containing the two varieties *indica* and *japonica* (*n*=38), red and black rice (*n*=15) and various commercial samples from Italy, Spain, India and the USA (*n*=8).

As examples, Figure 5 shows the levels of phosphate, *myo*-inositol and raffinose in the *lpa* mutants against the background of the levels from conventional rice samples. Except for the *lpa* mutant *Os-lpa*-XS110-1 grown at Hainan in 2005/2006, levels of *myo*-inositol in the wild-type and *lpa* mutants were within the natural range determined for the conventional brown rice samples. For phosphate and raffinose, both, wild-types and *lpa* mutants were within the natural ranges of the conventional "model"-rice database.

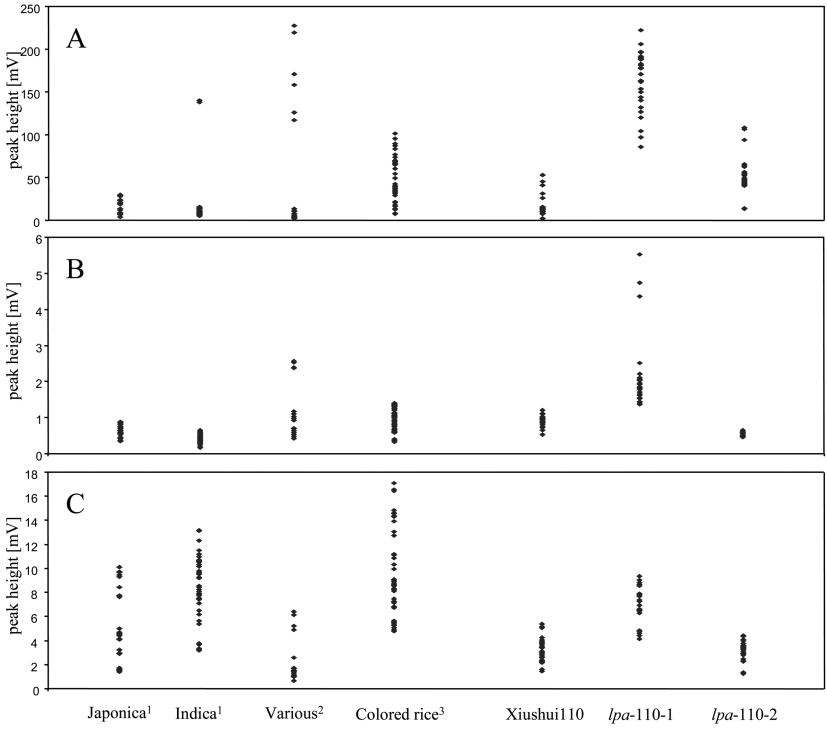

Figure 5. Variability of the metabolites phosphate (A), myo-inositol (B) and raffinose (C) in conventional brown rice, low phytic acid mutants and the corresponding wild-type. [1]Japonica (n=14) and Indica (n=24) varieties from China; [2]Varieties from Italy (n=4), Spain (n=2), India (n=1) and USA (n=1); [3]Red (n=4) and black (n=11) varieties from China.

Nutritional Aspects and Quality Assessment

The unbiased and non-targeted screening of a broad spectrum of metabolites also increases the probability to detect effects not intended by the mutation breeding process and thus may contribute to an adequate nutritional quality and

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safety analysis of the induced mutants. In this context, metabolites shown to be significantly different between *lpa* crop mutants and wild-types and additionally consistently present independent from environmental impacts, i.e. growing location and season, have to be further investigated. As example, the consistently increased raffinose content in the *lpa* mutant *Os-lpa*-XS110-1 has to be critically considered when the nutritional value of this mutant is assessed. However, potential unintended effects may not necessarily be unwanted. A comparable metabolite profiling procedure applied to a low phytic acid soybean mutant revealed significantly decreased contents of several oligosaccharides compared to the wild-type soybean (*82*) which would be a highly desirable trait from a nutritional point of view.

Food Processing

Metabolomics approaches are being employed to follow developmental changes in crops. An important stage in plant biology is represented by germination. Metabolic changes within this stage have been investigated by means of metabolite profiling in various crops including rice (*83*) and mung beans (*84*). In addition to the investigations of solely "natural" biological changes in crops during their life-cycle, metabolomics has become a rapidly emerging analytical approach in food science including food processing (*85*). As example, the process-related germination of barley is one of the most important applications in food technology.

Malting of Barley

Malting of barley represents an essential step within the brewing process of beer. This phase is characterized by numerous metabolic processes. Distinct and time-dependent alterations in metabolite levels are to be expected and metabolite profiling was used as an analytical tool to provide a comprehensive picture of these changes.

Barley grains were subjected to a micro-malting procedure involving steeping, germination and kiln-drying (*86*). Samples taken in the course of the malting process were subjected to the metabolite profiling procedure. This procedure allowed the parallel investigation of triglyceride-derived fatty acid methyl esters, hydrocarbons, free fatty acids, fatty alcohols, sterols, sugars, sugar alcohols, acids, amino acids and amines. Capillary gas chromatography analysis (Figure 6A) resulted in the detection of 587 distinct peaks of which 173 were identified on the basis of retention times and mass spectrometric data from authentic reference compounds and / or from mass spectral libraries.

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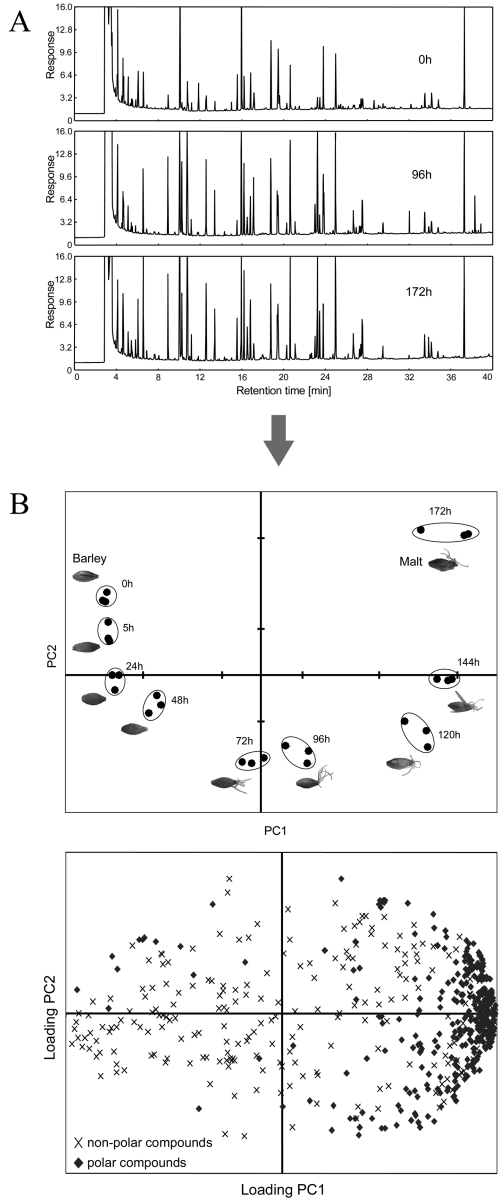

Figure 6. GC-based metabolite profiling (A) and multivariate data assessment by principal component analysis and investigation of corresponding factor loadings (B) of barley in the course of the malting process.

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As a first step, multivariate assessment of the metabolite profiling data was conducted by means of principal component analysis. PCA exhibited a clear clustering of the samples taken in the course of the malting process (Figure 6B). The progress of the malting procedure including germination and kiln-drying is described by a U-shape pattern. According to the described data assessment workflow, major drivers of the separation of samples from different stages of the malting process can be quickly examined by the PCA factor loadings (Figure 6B). Taken into consideration all covered lipophilic and hydrophilic metabolites, polar compounds were found to be major contributors to the separation along the first principal component, whereas predominantly the non-polar compounds were responsible for the separation along the second principal component. In addition to multivariate data analysis, univariate data assessment can be used for the analysis of single compounds. As examples, illustrations of a heatmap (Figure 7A) and quantitative analyses of selected compounds (Figure 7B) are given.

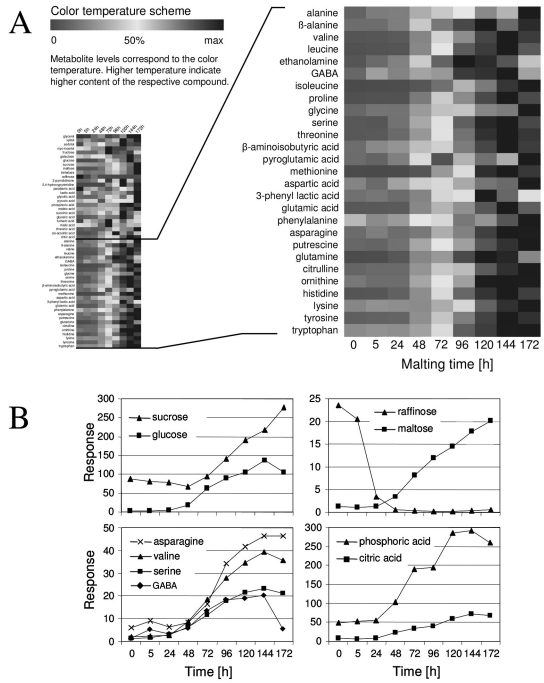

Figure 7. Univariate qualitative (A) and quantitative (B) analyses of metabolite profiling data obtained from barley the course of the malting process.

Metabolite profiling of barley grains in the course of the malting process in combination with appropriate multivariate and univariate data analyses confirm the potential of this technique to follow processing-related metabolic changes in cereals. Ideally, a set of biomarker could be developed representing the quality of the used cultivar and the applied malting conditions. Such marker metabolites

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might finally be correlated to the contents of the metabolites responsible for the technological properties and the nutritional quality of malted cereals.

Conclusions

Untargeted metabolite profiling as an approach for the simultaneous detection, identification and quantification of a broad spectrum of polar and non-polar constituents represents a suitable tool for the investigation of cereal grain metabolite phenotypes. Coverage of different impact factors, i.e. genetic background, breeding strategies (e.g. mutation breeding and genetic engineering), farming practices (e.g. organically and conventional farming) and environmental influences (e.g. growing location / season) on the grain metabolite profiles allows to assess the overall metabolic variation and thus provides comprehensive data for breeders to produce high-quality foods. In addition, metabolite profiling-based comparative investigations of crops grown under different environmental conditions enable consistent differences to be searched for in order to distinguish between natural variability and changes induced by different treatments such as genetic modification. In combination with an appropriate statistical data analysis, metabolite profiling therefore increases the probability of detecting effects not intended by the genetic modification and thus contributes to the safety assessment of such crops. In addition to its suitability to investigate plant breeding systems, metabolite profiling represents a useful tool to follow process-related metabolic changes in the course of barley malting. Due to the simultaneous coverage of a comprehensive set of metabolic data, metabolite profiling may assist in providing valuable information for further improvements of the quality of processed foods.

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Chapter 5

Anthocyanin-Pigmented Grain Products

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Anthocyanins play significant roles in human health and nutrition due to their positive functions in the areas of inflammation, diabetes, cancer, oxidative stress and ocular health. They also possess multiple uses as natural colorants, natural antioxidants and/or nutraceuticals. Thus research on the characterization of anthocyanins in grains in terms of extraction efficiency, composition, and antioxidant and health-enhancing properties is progressively growing. Processes for the development of anthocyanin-rich milling fractions and anthocyanin isolates based on dry milling, solvent extraction and column chromatography have also been developed. In general, a number of anthocyanin-pigmented grains such as blue or purple wheat, purple or blue corn, black or red rice and black sorghum hold a promise for the development of natural health products for food and nonfood applications. The current chapter discusses grain anthocyanins in terms of occurrence, composition, separation and health-enhancing properties, as well as their potential as natural colorants, functional food ingredients and/or dietary supplements.

Introduction

Functional foods and natural health products are rapidly being integrated into the corporate mainstream and increasingly being accepted by the public due to a steady demand from consumers for healthier foods and products. Grains constitute an important component of the human diet, and also contain several bioactive compounds including anthocyanins that have demonstrated

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health-enhancing and/or disease-preventing properties. Thus grains would offer immense opportunities for the development of a variety of functional foods and health products based on their content and composition of bioactive compounds. Anthocyanins have been incorporated into the human diet for centuries and have been used as traditional herbal medicines due to their multiple physiological abilities to treat health conditions such as hypertension, pyrexia, liver disorders, dysentery and diarrhea, urinary problems and the common cold (*1*). They are also of particular interest as natural colorants due to their ability to impart vibrant colors. Recently anthocyanin-rich grain ingredients are being incorporated into food products and such products require further research to demonstrate their physiological effects and efficacy.

Anthocyanins (Greek *anthos*, flower and Greek *kyanose*, blue) are the largest group of water-soluble pigments in the plant kingdom and belong to the family of compounds known as flavonoids which are part of an even larger group of compounds known as polyphenols (*2*). They are natural pigments responsible for the red, purple and blue hues in fruits, vegetables, flowers and grains, but also play important roles in plant physiology such as attractants for insect pollinators and seed dispersal (*3*). They are widely distributed in the human diet and the daily intake has been estimated at 12.5 mg/day in the United States (*4*). In this regard more research on anthocyanins in grain is required to develop anthocyanin-rich products as well as innovative processing technologies for the isolation of anthocyanins from grains. The current chapter discusses composition, characterization, and antioxidant and health-enhancing properties of anthocyanins found in grains. In addition, their potential as natural colorants and antioxidants, functional food ingredient and/or dietary supplements are highlighted. The fractionation technologies recently developed to isolate anthocyanins from blue or purple wheat are also discussed.

Chemical Structure

Anthocyanins or deoxyanthocyanins are glycoslyated, polyhydroxy or polymethoxy derivatives of 2-phenylbenzopyrylium that contains two benzoyl rings (A and B) separated by a heterocylic (C) ring (Figure 1). They comprise of anthocyanindin or aglycone and sugar moiety (nonacylated) or also contain organic acid (acylated). The sugar can be one molecule (monoglucoside) or two molecules (digluccoside). The majority of anthocyanins consumed (12.5 mg/day) in USA are nonacylated (77% of the total consumption) and monoglucoside (73% of the total consumption) (*4*). More than 400 individual anthocyanins have been identified in plants, but only six anthocyanidins are commonly found including cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin. The glycosides of the three non-methylated anthocyanidins (cyanidin, delphinidin and pelargonidin) are the most widespread in nature, being present in 80% of pigmented leaves, 69% of fruits and 50% of flowers (*5*). They are usually bound to a saccharide residue such as glucose, galactose, rhamnose or arabinose as 3-glycosides or 3,5-diglycosides. Cyanidin- and delphinidin-based anthocyanins account for 45 and 21% of the total anthocyanin consumed, respectively (*4*).

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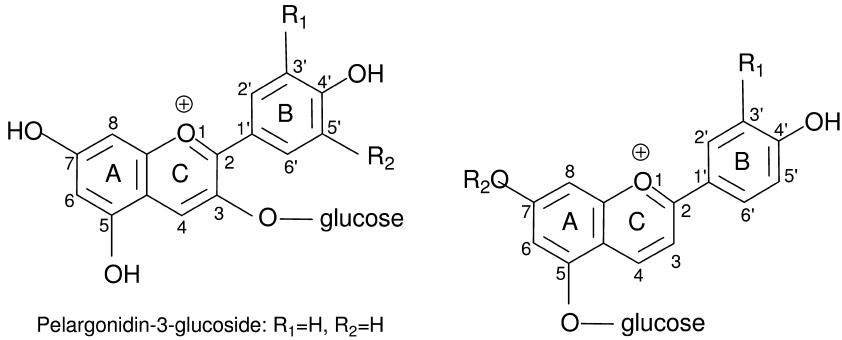

Pelargonidin-3-glucoside: R_1=H, R_2=H
Cyanidin-3-glucoside: R_1=OH, R_2=H
Delphinidin-3-glucoside: R_1=OH, R_2=OH
Peonidin-3-glucooside: R_1=OCH_3, R_2=H
Petunidin-3-glucoside: R_1=OCH_3, R_2=OH
Malvidin-3-rutinoside: R_1=OCH_3, R_2=OCH_3

Figure 1. Structures of the six common anthocyanidins in comparison with 3-deoxyanthocyanindins in glucoside form with glucose.

Color differences between anthocyanins are largely determined by the substitution pattern of the B-ring of the anthocyanidin, the pattern of glucosylation and the degree and nature of esterification of the sugars with aliphatic or aromatic acids as well as by extrinsic factors such as pH, temperature, type of solvent and the presence of co-pigments (*2*). In an aqueous solution at pH 1-3 the flavylium cation is red colored, at pH 5 the resultant carbinol pseudo base is colorless and at pH 7-8 the blue purple quinoidal base is formed (*5*). Color degradation can also occur in the presence of oxygen, light, ascorbic acid and/or metal ions and various enzymes. This is particularly important when incorporating them into foods and thus it is critical to determine shelf life of the product. Since anthocyanins are highly reactive compounds which readily degrade or react with other constituents in mixtures to form colorless or brown compounds, it is necessary to increase their stability in order to retain their color and functionality. Glucosylation primarily at the C-3 resulted in reduced maximum wavelength absorption and increased stability and solubility (*6*). Acylation of sugar residues with cinnamic (p-coumaric, caffeic, ferulic) or aliphatic (acetic, malonic, succinic) acids would further improve anthocyanin stability (*5*). Generally, 5-glucosylated structures degrade more easily than 3-glycosides followed by aliphatic acyl-anthocyanins and aromatic acyl derivatives (*6*). It is obvious that numerous factors would affect the use of anthocyanin products as a functional ingredient and/or food colorant, and such factors should be taken into account in the developmental process of these products.

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Analysis of Anthocyanins

Extraction

Extraction of anthocyanins from grains is usually carried out with acidified methanol or ethanol with the objective of obtaining the flavylium cation form, which is stable in a highly acid medium (*7*). No significant difference in extraction efficiency was observed between ethanol and methanol, but it is preferable to use ethanol since it is less toxic particularly for food use and clinical trials. In case the extract contains lipid materials, it is suggested to use an organic solvent such as hexane to eliminate any unwanted lipid-containing substances. Acid may cause partial hydrolysis of the acyl moieties in acylated anthocyanins, especially those with dicarboxylic acids such as malonic acid, and thus the use of weak acids is desirable, such as tartaric or citric acid to keep dicarboxylic substituents intact (*8*). Garcia-Viguera et al. (*9*) observed that extraction of anthocyanins from strawberries with 1% HCL in methanol or methanol:acetic acid:water (25:1:24, v/v/v) resulted in acetylation of anthocyanin sugars which was avoided when substituted with acetone.

The pH value is also known to significantly influence the color of anthocyanin extracts, absorbance readings and overall extractability. At lower pH (pH < 2), blue and purple wheat extracts exhibited a red to dark red color after extraction, while at a higher pH (pH>4) extracts displayed a yellow color (*7*). Additionally, absorbance readings increased with declining pH levels of the solvents tested (water, ethanol, and methanol). At higher pH levels (pH 5) extractability of anthocyanin is decreased by 94% in blue and purple wheat extracts compared to extraction at a lower pH (pH 1). Increasing temperature from 25 ºC to 60ºC during extraction of purple and blue wheat increased absorbance readings by 15%. Storage of blue and purple wheat anthocyanin extracts under cold conditions (4ºC) causes the extracts to become cloudy due to precipitation of the soluble proteins but has no significant effect on the absorbance readings.

Separation and Quantification

Analysis of anthocyanins is commonly performed by reversed-phase high performance liquid chromatography (RP-HPLC) equipped with photodiode array detector (PDA) because it permits simultaneous separation, identification and quantification without requiring excessive purity of the extracts. The separation columns are usually maintained at ambient temperature and the elution systems are binary using aqueous acidified solvents such as acetic acid, perchloric acid or formic acid in an organic solvent such as methanol or acetonitrile (*10*). The separated anthocyanin compounds are detected and measured at 525 nm and the identity of anthocyanins are based on corresponding retention times and ultraviolet-visible (UV-vis) spectra with those of pure authentic standards such as delphinine-3-glucoside, delphinidin-3-rutinoside, cyanidin-3-glucoside, cyanidin-3-galactoside, cyanidin-3-rutinoside, peonidin-3-glucoside, petunidin-3-glucoside, and pelargonidin-3-glucoside that are commonly found in grains and commercially available. Cyanidin chloride can be used as internal standard but must be

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prepared just before analysis due to its rapid degradation. The UV-vis absorbance spectrum of anthocyanins is useful in providing information on the nature of the anthocyanidin, glucosylation pattern and possibility of acylation (*11*). Anthocyanin compounds have a wide absorption range in the blue end of the visible spectrum with a maximum absorption observed in the 500-535 nm regions (*12*). The UV-vis data has the ability to confirm some of the anthocyanidins, i.e. the maximum absorption for pelargonidin-based compounds has been observed around 502-506 nm, cyanidin at 512-520 nm, peonidin at 517-520 nm, delphinidin at 525 nm, petunidin at 526-529 nm, and malvidin at 530 nm (*12*). More details about the analytical techniques for the separation of anthocyanins from grains have been described in our previous studies (*12*). Separation of anthocyanin compounds from black, blue, pink, purple and red barely, corn, rice and wheat are presented in Figures 2 and 3.

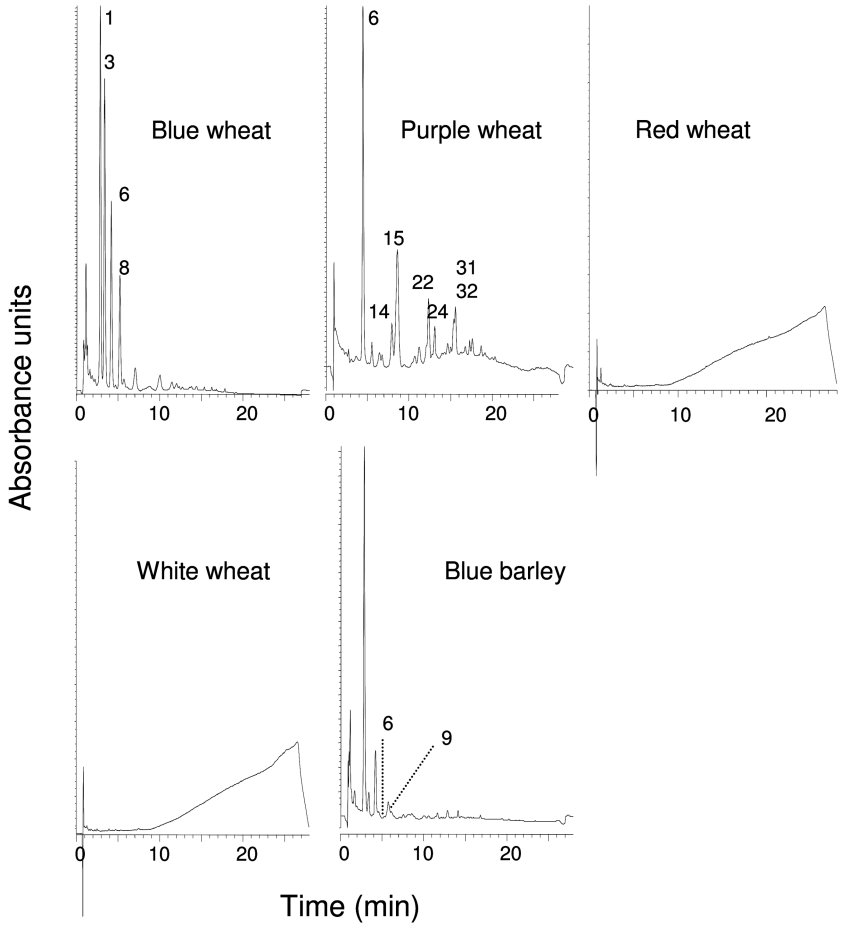

Figure 2. LC-UV/Vis chromatograms of anthocyanins in colored wheat and barley extracts separated on C18 column. The peak numbers show the major anthocyanins that are identified in Table 1. (Source: Abdel-Aal et al. 2006.)

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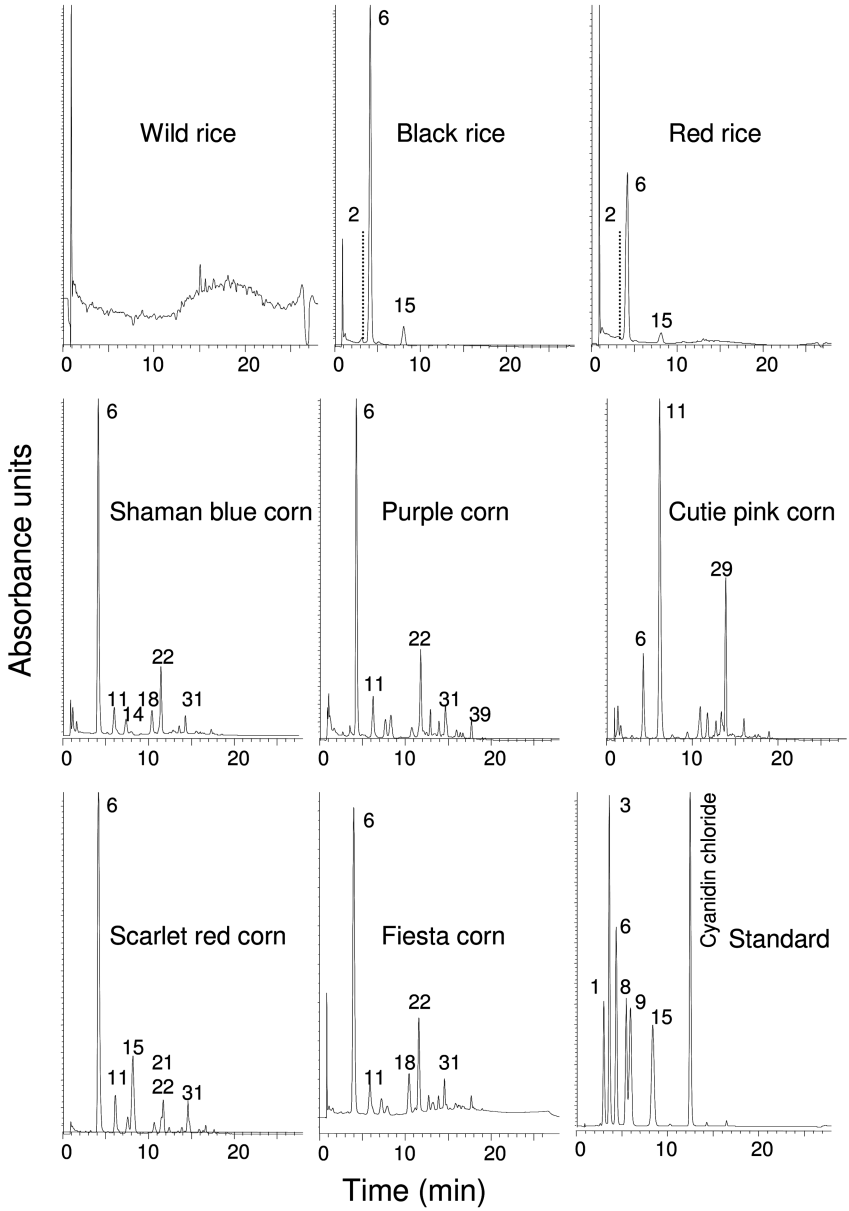

Figure 3. LC-UV/Vis chromatograms of anthocyanins in colored rice and corn extracts and anthocyanin standard mixture separated on C18 column. The peak numbers show the major anthocyanins that are identified in Table 1. (Source: Abdel-Aal et al. 2006.)

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Polarity of anthocyanidin/anthocyanin compounds is the most important factor that affects separation and retention time. Anthocyanidin compounds become more polar as the number of the hydroxyl group in the B-ring increases, thereby decreasing retention time and become more apolar as the number of methoxyl group increases. Thus, the observed order of elution of the six most common anthocyanidins when seprated on C18 column is in the following order delphindin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin. Sugar substitution increases the polarity with anthocyanins eluting at shorter retention times than their parent anthocyanidins with diglucosides usually eluting before the monoglycosides (*11*). However, the type of sugar moiety attached also influences the polarity depending on the type of sugar and its substation position. Acylation of the sugar moieties of the anthocyanins decreases polarity and is reflected in an increase of the retention time (malic > acetic > malonic > succinic) (*8*, *11*).

In a study 3 column temperatures (25, 38 and 50 °C) were investigated to determine separation efficiency of anthocyanins extracted from several colored grains particularly for those materials that exhibited complex compositions such as purple corn, pink corn and purple wheat (*12*). In order to prevent any thermal degradation during analysis of anthocyanins and to maintain column quality the highest temperature employed was 50 °C. When the anthocyanin standard mixture was run at the three temperatures, similar separations and responses were obtained indicating that no degradation occurred. In the case of sample extracts, poor separation (i.e., several co-eluted compounds) was obtained at 25 °C, whereas separation at 38 °C improved the resolution of anthocyanins. The LC chromatograms of anthocyanins separated at 38 °C for 13 grain extracts and standard mixture are presented in Figures 2 and 3. Separation of anthocyanins at 50 °C resulted in slight improvements for only those compounds eluted at longer retention time, i.e., separation of cyanidin succinylglucoside and peonidin succinylglucoside in purple corn. Thus it is advised to separate anthocyanin extracts at 38 °C, corn and purple wheat samples that have complex composition of anthocyanin were run at 38 and 50 °C for complete separation and better identification.

Capillary electrophoresis (CE) has also been used to separate ionic anthocyanin compounds by their charge (*13*). The use of CE in the separation of anthocyanins is fairly scarce, but promising due to the high hydro-solubility of these compounds. The CE method has been employed in quantitative determination of anthocyanins in wine as a RP-HPLC alternative (*14*), and variations on CE methods have been developed for acylated and non-acylated anthocyanin pigments and 3-glucoside derivatives (*15*).

Detection and Identification

Spectral properties have often been used for the characterization of anthocyanin pigments, in particular in the identification of anthocyanidin or aglycone type. For example UV and MS spectrum analyses have been employed to identify and quantify anthocyanins (*12*). As described earlier anthocyanin absorption spectra depends on pH. The maximum absorption at 520 to 540 nm in the visible region is the most common wavelength used in the spectrophotometric

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measurement of anthocyanins. Mass spectrometry (MS) permits anthocyanin identification by determining the mass of the molecular ions in the sample and of the fragments from the separation of these compounds through the application of higher ionization energies (*8*). Liquid chromatography-mass spectrometry (LC-MS) has been used to confirm the identity of anthocyanin compounds in plants and biological fluids. LC-MS combines the separation on LC system with the selectivity and sensitivity of the MS detector allowing the identification of individual components of a mixture of compounds such as plant extracts (*11*). Electrospray ionization mass spectrometry technique (ESI-MS) and nuclear magnetic resonance (NMR) have also been used for the identification of anthocyanins (*8*). ESI-MS is used for anthocyanin characterization in complex food matrices and is especially useful for detection of low level anthocyanin metabolites in human plasma (*10*). Low voltage and atmospheric pressure easily detects the positive charge of anthocyanins at low pH values since other potentially interfering compounds are not usually ionised. NMR is a powerful method used for structural elucidation of anthocyanins such as the acylated anthocyanins with rhamnoside derivatives found in some fruits like blackberries and minor compounds in wine analysis (*13*, *16*). The use of one dimensional and two dimensional ^{1}H and ^{13}C NMR spectroscopy with large signal suppression methods allow for reliable identification for ^{1}H and ^{13}C resonance signals of individual anthocyanins in deuterated methanol (CD_3OD) (*16*). These techniques can serve as a complement to the more widely used methods described.

The acidic methanol extracts of the black, blue, pink, purple, and red grains studied afforded complex mixtures that were characterized on the basis of UV/Vis and MS properties and the retention times of components separated by liquid chromatography. Wherever possible, confirmation of identity was achieved by the congruence of these properties with those of authentic anthocyanin standards. Of the 42 anthocyanin compounds observed in the anthocyanin-rich grains studied standards of only 9 were available for confirmation. The remaining components were tentatively identified on the basis of spectroscopic, spectrometric, and retention properties. The mass characteristics of anthocyanin pigments found in black, blue, pink, purple and red barely, corn, rice and wheat are presented in Table 1.

The assignment of a component to the anthocyanin class was first based upon UV/Vis spectra. Anthocyanins show a broad absorption band in the blue end of the visible spectrum with maxima in the 500 to 535 nm region (*17*). Absorption bands were also observed in the UV region about 277 to 284 nm, due to the aromatic ring structure, and occasionally another weak UV band in the region about 328 to 346 nm. The separated anthocyanin peaks from the standard mixture and blue wheat and purple corn extracts and their corresponding absorption spots are shown in the isoabsorbance plot (Figure 4) (*12*). This plot is useful in that it shows absorption bands in the visible and UV regions for each anthocyanin compound. Anthocyanins could also be characterized by fragmentation patterns arising from MS created by ESI in the positive (+ve) mode. They are typified by a central flavonoid ring aglycone structure, which is connected to a saccharide moiety; more than 539 possible combinations are known. The saccharides may be underivatized or have an attached acyl moiety. Different positional and structural

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isomer possibilities add to the complexity. A typical ESI +ve MS shows two ions: the protonated molecular ion $[M+H]^+$ and a fragment ion $[M+H-X]^+$ arising from loss of the saccharide moiety (*12*). However, since the anthocyanins have a natural residual positive charge, one observes a true molecular ion $[M]^+$ and a fragment ion $[M-X]^+$. The fragment ion is that of the underiviatized aglycone. There are six major aglycones generally observed with the associated fragment ions: pelargonidin, *m/z* 271; cyanidin, *m/z* 287; peonidin, *m/z* 301; delphinidin, *m/z* 303; petunidin, *m/z* 317; and malvidin, *m/z* 331. The value for X, based on the difference between the molecular ion and fragment, gives a clue to the nature of the saccharide functionality. The MS of the compound showing ions at *m/z* 287 and 449 suggests that the aglycone is cyanidin (*m/z* 287) and the difference of *m/z* 162 suggests a hexose. Two common hexoses would be glucose or galactose; ESI +ve MS cannot distinguish between them. Furthermore, the point of attachment to the aglycone also cannot be distinguished. For this, one could compare retention times and spectra with those of authentic standards. Several compounds with similar mass spectra but different retention times and spectroscopic properties were also found in colored grains. Such compounds require further analysis by other techniques such as NMR to confirm their identity. For examples, two isomers of cyanidin diglucoside were found in black and red rice at different retention times, and NMR would be capable to determine their identities.

Anthocyanins in Grains

Despite early studies on red pigmentation in wheat grains (*18*), research on characterization of anthocyanin pigments in grains has been increased only in the past few decades. This is likely due to the health benefits associated with anthocynins and the continuing increasing demand for antioxidant-rich wholegrain foods and natural colorants. Information on other pigments found in grains such as xanthophylls, carotenoids and flavones is also available. Anthocyanin pigments are found in the outer layers of the grain kernels, and perhaps are located in a specific tissue. For example, the purple pigments in purple wheat are located in the grain pericarp, while the blue pigments in blue wheat are concentrated in the aleurone layer (*19*). This division, however, is not entirely clear, and more research should aid in identifying their occurrence and functionality in the grains. Recently, the inheritance of the blue aleurone and purple paericarp in wheat were studied (*20*). For blue wheat cultivars, the blue pigment deposition began near the embryo and by the third harvest date the entire kernel was blue in color. For the purple pericarp wheats, pigment appeared near the apex at the third harvest date, with the entire grain pigmented by the fourth or fifth harvest date (25-28 or 30-33 days post anthesis). It appears that the blue aleurone trait is controlled by a single dominant gene which exhibits xenia phenotypes, whereas the purple trait fits a two locus model, segregating 11 purple:5 white ratio (*20*).

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Table 1. Anthocyanins Detected in Selected Black, Blue, Pink, Purple and Red Barley, Corn, Rice and Wheat and Their Spectroscopic and Mass Characteristics. Source: Abdel-Aal et al. 2006

RT (min)	*Compound #*	*Anthocyanin*	*λmax (nm)*	*Major ions (m/z)*	*Observation*
2.9	1	Dp-3-Glu	525	465, 303[a]	Confirmed
3.1	2	Cy-diGlu[1]	516	611, 287	Presumed
3.4	3	Dp-3-Rut	525	611, 303	Confirmed
3.5	4	Lt-Glu	490	433, 271	Presumed
3.8	5	Cy-3-Gal[b]	517	449, 287	Confirmed
4.1	6	Cy-3-Glu	517	449, 287	Confirmed
5.0	7	Cy-diGlu[1]	515	611, 287	Presumed
5.1	8	Cy-3-Rut	517	595, 287	Confirmed
5.7	9	Pt-3-Glu	526	579, 317	Confirmed
6.0	10	Cy-MalGlu[2]	512	535, 287	Presumed
6.2	11	Pg-3-Glu	502	433, 271	Confirmed
6.3	12	Cy-MalGlu[2]	516	535, 287	Presumed
7.1	13	Pt-3-Rut	529	625, 317	Confirmed
7.6	14	Cy-MalGlu[2]	516	535, 287	Presumed
8.3	15	Pn-3-Glu	517	463, 301	Confirmed
9.4	16	Pg-MalGlu[3]	504	519, 271	Presumed
10.0	17	Pn-Rut	519	609, 301	Presumed
10.6	18	Cy-SucGlu[4]	514	549, 287	Presumed
10.7	19	Pg-MalGlu[3]	506	519, 271	Presumed
11.5	20	Mv-Rut	530	639, 331	Presumed
11.2	21	Cy-MalGlu[2]	518	535, 287	Presumed
11.6	22	Cy-SucGlu[4]	518	549, 287	Presumed
12.2	23	Cy-SucGlu[4]	520	549, 287	Presumed
12.3	24	Pn-MalGlu	518	549, 301	Presumed
12.7	25	Pg-SucGlu[5]	503	533, 271	Presumed
12.8	26	Unknown	498	867, 705, 543, 417, 271	-
13.4	27	Pg-SucGlu[5]	506	533, 271	Presumed
13.5	28	Pg-MalGlu[3]	506	519, 271	Presumed
13.9	29	Pg-SucGlu[5]	502	533, 271	Presumed

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Table 1. (Continued). Anthocyanins Detected in Selected Black, Blue, Pink, Purple and Red Barley, Corn, Rice and Wheat and Their Spectroscopic and Mass Characteristics

RT (min)	*Compound #*	*Anthocyanin*	*λmax (nm)*	*Major ions (m/z)*	*Observation*
14.1	30	Pn-SucGlu[6]	518	563, 301	Presumed
14.6	31	Cy-SucGlu[4]	520	549, 287	Presumed
14.8	32	Pn-SucGlu[6]	520	563, 301	Presumed
15.8	33	Cy-Mal-SucGlu[7]	520	635, 287	Presumed
15.9	34	Pg-SucGlu[5]	506	533, 271	Presumed
16.4	35	Cy-Mal-SucGlu[7]	520	635, 287	Presumed
16.7	36	Pn-SucGlu[6]	520	563, 301	Presumed
17.3	37	Pg-Mal-SucGlu[8]	504	619, 271	Presumed
17.6	38	Pn-Mal-SucGlu	520	649, 301	Presumed
17.7	39	Cy-diSucGlu	520	649, 287	Presumed
17.8	40	Pg-Mal-SucGlu[8]	520	619, 271	Presumed
17.9	41	Pg-diSucGlu[9]	522	633, 271	Presumed
19.0	42	Pg-diSucGlu[9]	506	633, 271	Presumed
19.3	43	Pn-diSucGlu	520	663, 301	Presumed

Cy = cyanidin, Dp = delphinidin, Gal = galactoside, Glu = glucoside, Lt = luteolunidin, Mal = malonyl, Mv = malvidin, Pg = pelargonidin, Pn = peonidin, Pt = petunidin, Rut = rutinoside, Suc = succinyl.[1,2,3,4,5,6,7,8,9] Compounds with identical molecular mass within each superscript which suggest they are isomeric. [a] Molecular ion and fragment ion. [b] Only in standard.

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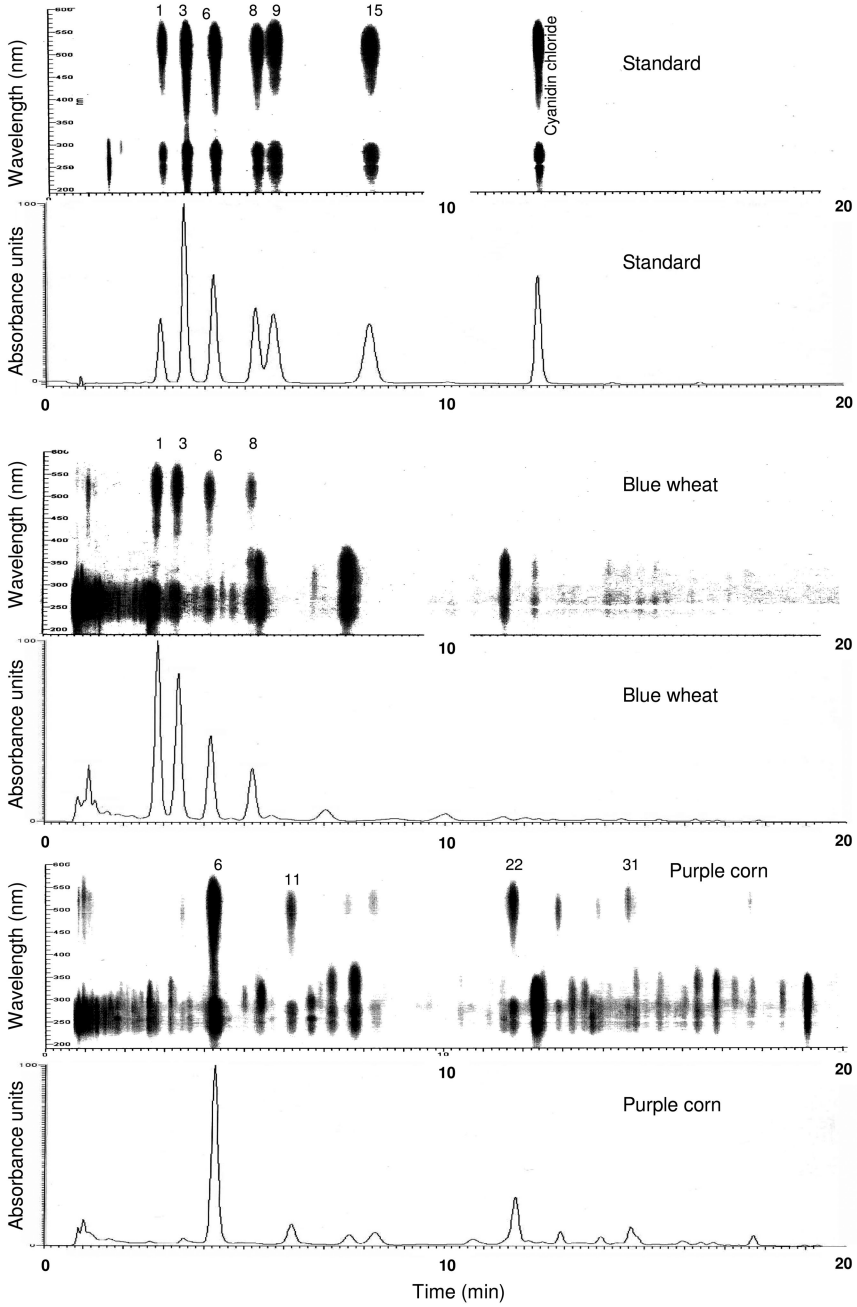

Figure 4. Typical illustration for isoabsorbance plots of anthocyanins separated from standard mixture, blue wheat and purple corn. The peak numbers show the major anthocyanins that are identified in Table 1. (Source: Abdel-Aal et al. 2006.)

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A large number of colorful grains or anthocyanin-pigmented grains are currently produced only in small amounts for small or niche markets, e.g. specialty foods or natural colorants. These include blue and purple wheat, blue, purple and red corn, black and red rice, and perhaps many other color grains. Blue, purple and red corn has been used in ornamentation due to their colourful appearance, and also has been used for making blue or pink tortilla. Purple wheat is crushed into large pieces and spread over the exterior of multi-grain bread (*21*). Red rice has been used as a food colorant in bread, ice cream and liquor (*22*). In addition, purple corn has been processed into a food colorant since 1977 (*23*). Other anthocyanin-pigmented black, blue or purple grains include barley, rye, triticale, oat, sorghum, millet, buckwheat, quinoa and amaranth (*24*). These color grains have a broad range of nutrients, antioxidants, characteristics and functionalities that would offer great opportunities for the development of a variety of functional foods and natural health products.

Early studies on anthocyanins in grains have shown that cyanidin 3-glucoside and peonidin 3-glucoside are the major anthocyanins in purple wheat and purple rye as detected by paper chromatography using rhubarb and plum extracts as standards (*25*, *26*). Using liquid chromatography, cyanidin 3-glucoside was found to be the most principal anthocyanin in purple wheat and the second common in blue wheat (*27*). In corn, cyanidin 3-glucoside, cyanidin-3-(6″-malonylglucoside) and cyanidin-3-(3″,6″-dimalonylglucoside) were the major anthocyanins with cyanidin being the main aglycone or anthocyanidin, accounting for 73 to 87% of the total (*28*). Similar anthocyanin composition was also found in corn flowers (*29*). Black rice contained a wide range of total anthocyanin content with cyanidin 3-glucoside being the most common anthocyanin (0.0-470 mg/100g) in most of the ten varieties studied, whereas peonidin 3-glucoside (0.0-40 mg/100g) was the second dominant anthocyanin (*30*). Black sorghum possessed the highest total anthocyanin content among selected black, brown and red sorghum cultivars with luteolinidin and apigeninidin being the major deoxyanthocyanidins in black sorghum accounting for 50% of the total anthocyanins (*31*, *32*). These studies show substantial differences in anthocyanin content and composition between grains which would influence their use as natural colorants or functional ingredients. The sorghum 3-deoxyanthyanin pigments (Figure 1) were found to be more stable to pH-induced color loss than the anthocynins commonly found in fruits and vegetables and hold a promise as potential food colorants (*32*). For additional information about 3-deoxyanthocyanins in sorghum see chapter on sorghum flavonoids. The composition of anthocyanins in black and red rice, blue and purple wheat and blue barley is presented in Table 2 and blue, pink, purple, red and multicoloured corn is given in Table 3.

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Table 2. Average Concentration of Anthocyanins (µg/g) in Black and Red Rice and Blue and Purple Wheat and Blue Barley. Source: Abdel-Aal et al. 2006

Antho-cyanin	*Com-pound #*	*Black rice*	*Red rice*	*Blue wheat*	*Purple wheat*	*Blue barley*
Dp-3-Glu	1	-	-	56.5 ±4.6	-	-
Cy-diGlu[1]	2	71.8 ±2.0[a]	1.7 ±0.1		-	-
Dp-3-Rut	3	-	-	49.6 ±2.4	-	-
Cy-3-Glu	6	2013 ±57.1	14.0 ±0.3	20.3 ±1.5	4.0 ±0.1	1.2 ±0.0.02
Cy-diGlu[1]	7	16.7 ±0.2	tr	-	-	-
Cy-3-Rut	8	19.9 ±0.4	1.3 ±0.1	16.8 ±0.8	-	-
Pt-3-Glu	9	-	-	2.2 ±0.1	-	2.9 ±0.1
Pt-3-Rut	13	-	-	4.5 ±0.2	-	-
Cy-Mal-Glu	14	-	-	-	1.2 ±0.1	-
Pn-3-Glu	15	162.1 ±4.6	2.5 ±0.1		2.1 ±0.1	
Pn-Rut	17	-	-	1.2 ±0.1	-	-
Cy-SucGlu[2]	18	-	-	-	0.6 ±0.04	-
Mv-Rut	20	-	-	2.0 ±0.1	-	-
Cy-SucGlu[2]	22	-	-	-	1.1 ±0.2	-
Pn-MalGlu	24	-	-	-	0.6 ±0.2	-
Cy-SucGlu[2]	31	-	-	-	1.2 ±0.1	-
Pn-SucGlu[3]	32	-	-	-	0.9 ±0.1	-
Pn-SucGlu[3]	36	-	-	-	0.6 ±0.1	-
Pn-Mal-SucGlu	38	-	-	-	0.5 ±0.1	-
Total	-	2283.5	21.8	153.1	12.8	4.1

Cy = cyanidin, Dp = delphinidin, Glu = glucoside, Mal = malonyl, Mv = malvidin, Pn = peonidin, Pt = petunidin, Rut = rutinoside, Suc = succinyl, tr = trace, <0.1 µg/g.[1,2,3] Compounds with identical molecular mass within each superscript which suggest they are isomeric. [a] Mean ± SD.

In a study on anthocyanins in blue wheat a population of 160 breeding lines was found to contain total anthocyanin content ranging 34.6-507.2 µg/g with a mean value of 183.0 µg/g and standard error of 7.6 (*7*). The concentration

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of anthocyanins in the experimental lines was normally distributed, and the absorbance reading of anthocyanin extracts was significantly correlated with the grain color values measured with a hunterlab colorimeter. The pigments extracts were stable when stored in fridge at 4°C for up to 8 weeks with the exception of the appearance of cloudiness after the first week. Total anthocyanin content was also found to vary significantly between black, blue, pink, purple, red and white wheat, barley, corn and rice wholegrain flours (*12*). Black rice with an average of 3276 μg/g was found to possess the highest total anthocyanin content among the color grains tested. This is about 35 times higher than that of red rice (94 μg/g). Wild rice had a very small concentration of total anthocyanin content which may belong to other group(s) of pigments since no anthocyanin peaks were detected by LC analyses (Figure 2). Significant differences in the concentration of total anthocyanins were also found between black, brown and red sorghum (*31*) as well as among black rice (*30*) cultivars. Black rice had a wide range of total anthocyanins depending upon cultivar, whereas black sorghum exhibited the highest total anthocyanins in a selection of black, brown and red sorghum cultivars.

Eight corn grains exhibiting blue, pink, purple and red attractive colors were found to contain a wide range of total anthocyanin content as low as 51 μg/g and as high as 1277 μg/g (*12*). Purple corn had the highest concentration followed by sweet scarlet red corn and Shaman blue corn. At present, most of the colored corn is used in ornamentation due to its colorful appearance and only a small amount is being used in making natural blue or pink tortilla. Blue wheat had an average total anthocyanin content of 212 μg/g (*12*) which is higher than that previously reported (139-164 μg/g) (*27*). Anthocyanin contents were significantly influenced by growing conditions and environment in blue and purple wheats and the environmental effect was much stronger in the purple wheat due to the pigment location in the outer pericarp or fruit coat (*27*). Thus, anthocyanins in purple wheat are more prone to environmental effects. Purple wheat contained lower total anthocyanin content compared to blue wheat and there were significant differences between the two purple wheat cultivars. Red and white wheat cultivars exhibited small concentrations of total anthocyanin content and no significant differences were observed between them. The LC analysis of red and white wheat extracts showed an absence of anthocyanin compounds in these wheats (Figure 2).

Fractionation of rice and wheat kernels into bran and flour fractions by abrasive or roller milling was able to concentrate anthocyanin pigments in the bran fractions (*12*). In fact, the pigments were concentrated by about 8, 6 or 4 times in black rice, red rice or blue wheat bran fraction, respectively. However, the recovery of pigments in the bran fractions obtained by 1 min abrasion in the case of rice or in the shorts or fine bran (>250μm <420μm) in the case of wheat was low, ranging from 25 to 28%. The yield of these fractions was also low and ranged from 3 to 7%. In black, brown and red sorghum, the bran fraction (15% yield) contained 3-4 times higher anthocyanin content than the whole grains but no data was reported on anthocyanin recovery (*31*, *32*). Addition of 4 min abrasion in the case of rice or adding the coarse bran (>420μm) in the case of wheat increased the pigment recovery to 92, 88 and 80% in the combined bran fractions obtained from black rice, red rice and blue wheat, respectively

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(*12*). The average yield of the combined bran fractions was also substantially increased, accounting for approximately 22, 16 and 30% of the total fractions for black rice, red rice and blue wheat, respectively. The high yield and recovery of anthocyanins in these grain fractions support the use of dry milling processes as physical fractionation technologies to produce anthocyanin-rich fractions for use as functional food ingredients or natural colorants.

Table 3. Average Concentration of Anthocyanins (µg/g) in Blue, Pink, Purple, Red and Fiesta Multicolored Corn. Source: Abdel-Aal et al. 2006

Anthocyanin	*Compound #*	*Sheman blue*	*Cutie pink*	*Purple*	*Scarlet red*	*Fiesta corn*
Lt-Glu	4	-	-	31.0 ±1.4	-	1.2 ±0.1
Cy-3-Glu	6	110.2 ±5.1[a]	7.8 ±0.4	298.9 ±14.4	284.5 ±11.0	47.1 ±2.2
Cy-3-Rut	8	1.1 ±0.03	-	-	-	-
Cy-MalGlu[1]	10	1.5 ±0.03	-	-	-	-
Pg-3-Glu	11	12.1 ±0.3	39.8 ±2.1	55.3 ±2.6	27.7 ±1.9	6.8 ±0.3
Cy-MalGlu[1]	12	2.9 ±0.1	0.6 ±0.03	3.6 ±0.3	4.2 ±0.4	1.2 ±0.1
Cy-MalGlu[1]	14	13.2 ±0.7	1.2 ±0.1	32.0 ±0.5	16.8 ±0.9	3.9 ±0.2
Pn-3-Glu	15	2.3 ±0.1	0.7 ±0.1	27.3 ±1.2	58.7 ±2.5	2.2 ±0.1
Pg-MalGlu[2]	16	0.8 ±0.1	1.3 ±0.1		0.7 ±0.1	0.3 ±0.04
Cy-SucGlu[3]	18	12.2 ±0.7	1.1 ±0.1	14.7 ±0.4	11.3 ±0.5	8.9 ±0.2
Pg-MalGlu[2]	19	1.2 ±0.1	4.9 ±0.3	5.5 ±0.3	2.1 ±0.1	0.5 ±0.02
Cy-MalGlu[1]	21	6.9 ±0.4	0.4 ±0.01	34.8 ±1.7	20.2 ±1.1	4.7 ±0.1
Cy-SucGlu[3]	22	30.9 ±1.6	3.3 ±0.2	101.6 ±7.1	30.1 ±1.3	12.6 ±0.3
Cy-SucGlu[3]	23	-	-	11.2 ±1.1	4.8 ±0.2	-
Pg-SucGlu[4]	25	0.3 ±0.03	2.4 ±0.2	-	0.6 ±0.1	0.2 ±0.02
Pg-SucGlu[4]	27	1.4 ±0.1	3.5 ±0.1	-	1.8 ±0.2	1.6 ±0.1

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Table 3. (Continued). Average Concentration of Anthocyanins (μg/g) in Blue, Pink, Purple, Red and Fiesta Multicolored Corn

Anthocyanin	*Compound #*	*Sheman blue*	*Cutie pink*	*Purple*	*Scarlet red*	*Fiesta corn*
Pg-MalGlu[2]	28	-	0.5 ±0.02	21.2 ±1.1	1.6 ±0.1	-
Pg-SucGlu[4]	29	2.6 ±0.2	15.9 ±0.7	22.1 ±1.2	2.1 ±0.2	1.9 ±0.2
Pn-SucGlu[5]	30	-	-	12.3 ±0.5	3.7 ±0.1	-
Cy-SucGlu[3]	31	12.9 ±0.7	1.4 ±0.2	97.1 ±5.1	43.5 ±2.1	3.9 ±0.3
Pn-SucGlu[5]	32	1.1 ±0.1	-	14.5 ±0.7	7.1 ±0.4	0.8 ±0.1
Cy-Mal-SucGlu[6]	33	2.1 ±0.2	-	25.3 ±1.3	4.8 ±0.3	-
Pg-SucGlu[4]	34	2.3 ±0.2	3.6 ±0.2	23.9 ±1.4	4.3 ±0.3	-
Cy-Mal-SucGlu[6]	35	2.6 ±0.2	-	35.1 ±2.1	5.1 ±0.2	-
Pn-SucGlu[5]	36	-	-	13.5 ±0.7	9.5 ±0.5	-
Pg-Mal-SucGlu[7]	37	-	0.9 ±0.1	3.7 ±0.2	0.6 ±0.1	-
Cy-diSucGlu	39	4.6 ±0.2	1.5 ±0.1	57.6 ±3.1	8.7 ±0.4	2.1 ±0.1
Pg-Mal-SucGlu[7]	40	-	-	3.1 ±0.1	0.9 ±0.1	-
Pg-diSucGlu[8]	41	-	0.4 ±0.03	-	-	-
Pg-diSucGlu[8]	42	-	2.1 ±0.2	14.3 ±0.1	0.8 ±0.1	-
Pn-diSucGlu	43	-		5.6 ±0.3	2.3 ±0.1	-
Total	-	225.2	93.3	965.2	558.5	100.0

Cy = cyanidin, Glu = glucoside, Lt = luteolunidin, Mal = malonyl, Pg = pelargonidin, Pn = peonidin, Pt = petunidin, Rut = rutinoside, Suc = succinyl.[1,2,3,4,5,6,7,8] Compounds with identical molecular mass within each superscript which suggest they are isomeric. [a] Mean± SD.

Fifteen color grains exhibited diverse anthocyanin compositions ranging from only a few pigments or simple profile such as in black rice, red rice and blue barley (Figure 3), intermediate profile such as blue and purple wheat (Figure 2) to a complex profile such as blue, pink, purple and red corn (Figure 3) (*12*). Cyanidin 3-glucoside was the most abundant anthocyanin in black and

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red rice, accounting for 88 and 67% of the total anthocyanins, respectively. Peonidin 3-glucoside came second after cyanidin 3-glucoside in black and red rice, whereas cyanidin diglucoside was the third major anthocyanin in both rice grains. Two isomers of cyanidin diglucoside were observed in black and red rice, which could be positional or structural isomers due to differences in hexose type and/or position. Ryu and others (*30*) found two main anthocyanins in ten black rice varieties in which cyanidin 3-glucoside is the most common (0.0-470 mg/100g), whereas peonidin 3-glucoside (0.0-40 mg/100g) is the second. The anthocyanin composition of blue wheat differed from that of purple wheat. The main anthocyanin found in blue wheat is delphinidin 3-glucoside, being about 37% of the total anthocyanins. Delphinidin 3-rutinoside was the second dominant anthocyanin at 32% of the total anthocyanins. Delphinidin is the main aglycone in blue wheat, being about 69% of the total anthocyanidins. In purple wheat, 10 anthocyanin compounds were observed at small concentration with cyanidin 3-glucoside and peonidin malonylglucoside being the main ones. Cyanidin 3-glucoside is the principal anthocyanin in purple wheat.

Blue, pink, purple, red and multicoloured corn exhibited complex anthocyanin composition having from 18 to 27 compounds. The highest number of anthocyanins (27 compounds) was found in Scarlet red corn, whereas the highest amount was observed in purple corn (965 μg/g), with 25 anthocyanins. Cyanidin 3-glucoside was the most common anthocyanin in colored corn, except for pink corn, accounting for 51, 49, 47 and 31% in red, blue, multicoloured and purple corn, respectively. In pink corn, the major anthocyanin was pelargonidin 3-glucoside at 43% of the total anthocyanins. The second abundant anthocyanin was different in corn being cyanidin 3-succinylglucoside in blue, purple and multicolored corn, cyanidin 3-glucoside in pink corn and peonidin 3-glucoside in red corn. Moreno and others (*28*) reported that cyanidin 3-glucoside, cyanidin-3-(6″-malonylglucoside) and cyanidin-3-(3″,6″-dimalonylglucoside) are the major anthocyanins with cyanidin being the main aglycone accounting for 73 to 87% of the total anthocyanins in purplish-red corn. The majority of the anthocyanins found in colored corn were in acylated form having malonyl or succinyl moieties and several isomers. For example, isomers of cyanidin malonylglucoside, pelargonidin malonylglucoside, cyanidin succinylglucoside and pelargonidin succinylglucoside were found in blue, pink, purple and red corn, whereas isomers of pelargonidin succinylglucoside and cyanidin malonylsuccinylglucoside were observed in blue, purple and red corn. Pelargonidin malonylsuccinylglucoside and pelargonidin disuccinylglucoside isomers were found only in pink, purple and red corn but not in blue corn. Since glucose is the most common sugar in corn anthocyanins, it was presumed to be the hexose in those pigments. However, it is possible that other hexoses such as galactose may also be present in colored corn. In addition, sugar is commonly attached at position 3 which may suggest that most of the identified anthocyanins may have the hexose at position 3. A deoxyanthocyanin, luteolunidin glucoside, was also detected in purple and Fiesta multicoloured corn. This deoxyanthocyanin along with apigeninidin or apigeninidin glucoside were the major deoxyanthocyanins in black sorghum accounting for 50% of total anthocyanins (*32*).

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Separation of Anthocyanin-Rich Milling Fractions and Isolates

Anthocyanin-pigmented grains such as black rice, blue wheat and purple corn hold a promise as functional food ingredients, natural colorants, natural antioxidants and/or dietary supplements (*12*). Anthocyanin pigments are located in certain layers of the grain kernel which could be concentrated and separated into anthocyanin-rich fractions. In wheat, the blue pigments are located in the aleurone layer, whereas the purple pigments are concentrated in the pericarp layers (*7*, *19*). In corn, the highest concentration of anthocyanin pigments was found in the pericarp, whereas the aleurone layer contained small concentrations (*28*). Thus anthocyanins can be concentrated in the bran fractions of these grains using dry milling, and also can be further processed to isolate anthocyanins by a chemical process. Figure 5 depicts a process for the preparation of blue wheat wholegrain flour, blue wheat bran and anthocyanin powder, as well as fractionation of anthocyanin powder into individual anthocyanin compounds (*33*). In the developed process the bran fraction was used as a highly-anthocyanin substance to isolate anthocyanin powder. The blue wheat fine bran fraction or shorts had approximately 818 µg/g total anthocyanins whereas the coarse bran fraction contained 496 µg/g (*12*). Despite the high content of anthocyanins in the shorts fraction compared with the coarse bran, both fine and coarse bran fractions were combined to increase the yield of bran (26%) compared with only 7% yield for the shorts fraction and used in the isolation process. Dry milling such as abrasive or roller milling process is used to concentrate anthocyanin in the bran fraction, and then the highly-anthocyanin bran was subjected to solvent extraction and column chromatography to isolate and purify anthocyanin extracts. Following extraction with ethanol, the extracts were purified to remove sugars and proteins and evaporated to remove ethanol prior to freeze drying to obtain anthocyanin powder. Anthocyanin powder had an exceptionally high content of total anthocyanins at level of 3987 or 3378 mg/100g as determined by colorimetry or HPLC, respectively. This represents a 243-fold increase over the original blue wheat wholegrain flour (13.9 mg/100g) based on total anthocyanins measured by HPLC. The powder is extremely high in anthocyanins compared with blue wheat milling fractions and would hold the potential to be a natural colorant or natural health product.

The anthocyanin pigments in blue wheat have been reported to include four main compounds, namely delphinidin-3-glucoside, cyanidin-3-glucoside, delphinidin-3-rutinoside and cyanidin-3-rutinoside (*12*, *33*). In addition to the main anthocyanin compounds, blue wheat extracts contained low concentrations of petunidin-3-rutinoside, petunidin-3-glucoside, malvidin-3-rutinoside and peonidin-3-rutinoside. The identity of these pigments was confirmed based on the congruence of UV/Vis and MS data as described earlier. The concentrations of anthocyanins were very low in the white flour fraction and extremely high in the powdered product (Table 4). Delphinidin-3-glucoside constituted the highest proportion at 44-46%, followed by cyanidin-3-glucoside (28-29%), delphinidin-3-rutinoside (21-22%) and cyanidin-3-rutinoside (1.8-1.9%). In another study using the dark blue-grained wheat cv. Hedong Wumai, a different mix of anthocyanin pigments (cyanidin-3-glucoside, cyanidin-3-galactoside,

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pelargonidin-3-glucoside and peonidin-3-glucoside) were observed with cyanidin-3-glucoside being the predominant pigment (*34*). This variation in anthocyanin composition in blue wheat may be genotype dependent.

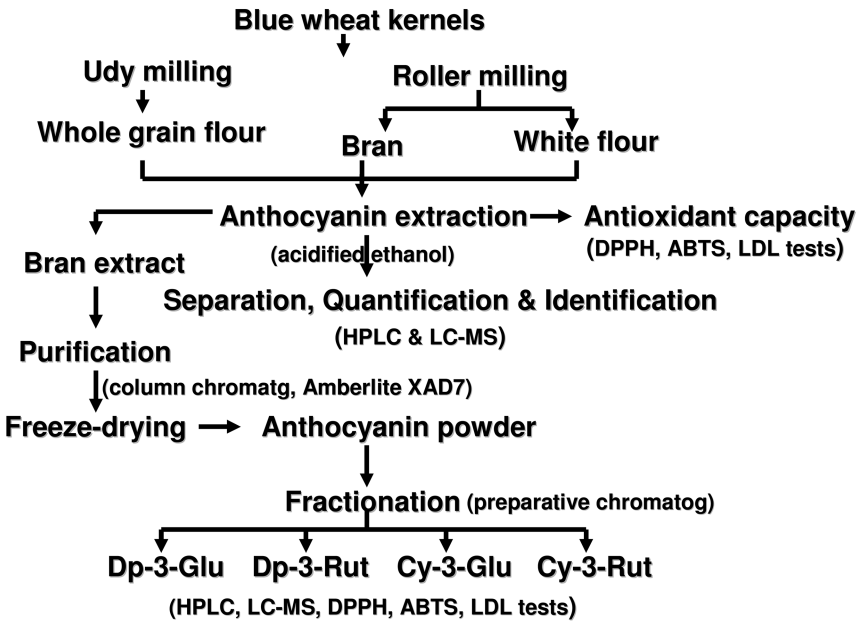

Figure 5. Flowchart showing preparation of blue wheat anthocyanin powder and anthocyanin compounds as well as their analyses and antioxidant properties. (Source: Abdel-Aal et al. 2008.)

Table 4. Average Concentration of Major Anthocyanin Compounds in Blue Wheat Products Determined by HPLC. Source: Abdel-Aal et al. 2008

Product	*Dp-3-Glu*	*Cy-3-Glu*	*Dp-3-Rut*	*Cy-3-Rut*
White flour (μg/g)	11.1 ±0.43[a]	6.9 ±0.29	5.2 ±0.24	0.45 ±0.03
Whole grain (μg/g)	57.1 ±2.7	37.6 ±1.5	27.8 ±0.8	2.3 ±0.07
Bran fraction (μg/g)	135.2 ±3.1	85.7 ±3.2	65.1 ±2.2	5.3 ±0.17
Anthocyanin powder (mg/g)	14.13 ±0.43	9.03 ±0.29	6.84 ±0.27	0.55 ±0.04

Cy, cyanidin; Dp, delphinidin; Glu, glucoside; Rut, rutinoside. [a] Mean ± SD, n = 3.

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Health-Enhancing Properties

Anthocyanins exhibit a wide range of therapeutic effects that have been demonstrated in various systems including *in vitro* system, animal experiments and human trials. Studies have shown that anthocyanin extracts can improve sight acuteness (*5*, *35–37*), display antioxidative and radical-scavenging activity (*33*, *38–42*) and to act as chemo-protective agents (*43–46*). They also possess antidiabetic properties such as lipid lowering (*47–50*), insulin secretion (*51–53*) and vasoprotective effects (*54*). Detailed information about the role of anthocyanins in human health and nutrition is recently reviewed (*55*).

Antioxidant Properties

In general, numerous *in vitro* studies have shown that anthocyanins and anthocyanidins are powerful antioxidants against free radicals, oxidation of human LDL cholesterol and lipids. Their powerful antioxidant properties are comparable and sometimes even higher than that of well-known antioxidants such as α-tocopherol, Trolox, ascorbic acid, BHT, BHA and rutin. However, the mechanisms by which anthocyanins exert their potent antioxidant capacities are complex and remain unclear due to the multitude of factors that affect their antioxidant properties. It is proposed that radical scavenging capacity of flavonoids can be maximized if there is a free hydroxyl group in the ring B. This may explain the higher radical scavenging capacity of many of the anthocyanidins compared to the glycosides in the DPPH radical scavenging test. In lipid-containing systems such as human LDL cholesterol or MeLo, glucosylation increases the polarity of the compound, thus affecting the access of the antioxidant to the lipid phase. This is supported by Kahkonen and Heinonen (*40*) who found that diglycosides of cyanidin or 3,5-diglucosides to be less active in inhibiting LDL oxidation than the corresponding 3-glucosides. However, in MeLo system, glucosylation either increased (petunidin and pelargonidin), remained unchanged (malvidin and cyanidin) or decreased (delphinidin and peonidin) their activity (*40*).

Only limited data is available on antioxidant properties of anthocyanins in humans. Studies have shown that following consumption of anthocyanins, serum antioxidant status is significantly increased after 4 to 24 hr post-consumption (*56*, *57*). Mazza *et al.* (*56*) observed a direct correlation of the appearance of total anthocyanins in the serum after participants consumed 100 g of blueberry supplement (1.2 g of anthocyanins) with an increase in serum antioxidant capacity ($p<0.01$) as measured by the ORAC assay. Antioxidant capacity in human serum has also been measured using various methods following consumption of strawberries (240 g), spinach (294 g), red wine (300 mL) or vitamin C (1250 mg) in eight elderly women (*57*). Total antioxidant capacity of serum increased significantly by 7-25% during 4 hr period after consumption of red wine, strawberries, vitamin C or spinach. The total antioxidant capacity of urine determined by ORAC (oxygen radical absorbance capacity) test also increased ($p<0.05$) for all the treatments except red wine, with the largest increase for vitamin C (44.9%). The observed antioxidant protective effects against ROS

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and increased in human serum indicate that the potent antioxidant activity of anthocyanins is not limited to *in vitro* studies, but is also observed *in vivo* suggesting that dietary consumption of anthocyanins is beneficial to human health.

Anthocyanin powder and individual anthocyanin compounds isolated from blue wheat bran (Figure 5) showed exceptionally high scavenging capacities (*33*). Anthocyanin powder had a DPPH scavenging capacity 42-fold higher than blue wheat bran. There were significant differences between anthocyanin compounds in their DPPH scavenging capacity. Cyanidin-containing anthocyanins had higher DPPH scavenging capacity compared with delphinidin-based anthocyanins. This finding emphasizes the importance of the molecular structure of anthocyanins in antioxidant properties and perhaps bioactivity. They were also exceptionally high in ABTS scavenging capacity compared with the blue wheat milling products. Anthocyanin powder had an ABTS scavenging capacity 54-fold higher than blue wheat bran. In addition, there were significant differences between anthocyanin compounds in their ABTS scavenging capacity. Again, cyanidin-containing anthocyanins exhibited higher ABTS scavenging capacity compared with delphinidin-based anthocyanins. This trend is similar to that obtained with DPPH scavenging capacity. The sugar moiety was also found to influence ABTS scavenging capacity of anthocyanin compounds. In this respect, rutinose-containing anthocyanins showed higher scavenging capacity compared to glucose-containing anthocyanins.

Blue wheat anthocyanin powder and anthocyanin compounds were also remarkably high in their ability to inhibit copper-induced human LDL cholesterol oxidation compared with the blue wheat milling products (*33*). For instance, anthocyanin powder exhibited inhibition capacity 25-fold higher than blue wheat bran and was comparable to that of the isolated anthocyanin compounds. Significant differences between anthocyanin compounds in inhibition capacity of LDL oxidation were also observed. Contrary to the free radical scavenging capacity, cyanidin-containing anthocyanins possessed lower inhibition capacity against LDL oxidation compared with delphinidin-based anthocyanins. The inhibition of LDL oxidation reaction is based on hydrogen atom transfer that is different from DPPH or ABTS reaction (*58*). The aglycone delphinidin has a structure similar to cyanidin except that it contains one more hydroxyl group at the C5 position (Figure 1). This additional hydroxyl group would change the release of hydrogen ions and thus the hydration constant (pK_H). Hydration constants, color and antioxidant properties of cyanidin-based anthocyanins were influenced by their structure, i.e. site of glucosylation as well as type and degree of acylation with cinnamic acid (*59*). The data demonstrate the presence of significant differences between the isolated blue wheat anthocyanin pigments in terms of free radicals scavenging capacity and inhibitory effects of oxidation of human LDL cholesterol in vitro. Barley and barley milling fractions were also found to possess good inhibition effects against copper-induced human LDL cholesterol oxidation (*60*, *61*). DPPH and ABTS scavenging reaction and LDL oxidation reaction data clearly demonstrate that blue wheat anthocyanins are potent antioxidants. In addition, composition of anthocyanins such as type of aglycone and sugar had a significant impact on antioxidant properties. Anthocyanins having different

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aglycones and sugar moieties were also found to have different responses in term of bioavailability and health effects (*62–64*). In addition, acylated anthocyanins also showed different stability and bioavailability compared with non-acylated anthocyanins (*65*, *66*).

Ocular Effects

The effect of anthocyanins on vision is one of the first reported health benefits (*5*). The idea that bilberry can be used to enhance night vision arose from reports of the British Royal Air Force aviators eating bilberry jam to improve their night vision during World War II (*67*). The majority of the data on the effect of anthocyanins on vision were conducted under rigorous experimental conditions, including randomized, double-blind, placebo-controlled and cross-over trials. Jang and others (*68*) determined the ability of bilberry extract to modulate adverse effects of A2E on retinal pigment epithelial cells *in vitro*. A2E is an auto-fluorescence pigment that accumulates in retinal pigment epithelial cells with age and can mediate a detergent-like perturbation of cell membranes and light-induced damage to the cell. This is significant since it is generally accepted that age-related macular degeneration begins with the death of retinal pigment epithelial cells, the degeneration of photoreceptor cells followed soon after by the loss of vision (*68*). Bilberry extracts were able to suppress the photooxidation of pyridinium disretinoid A2E by quenching singlet oxygen. In a double-blind, placebo-controlled, cross-over study, Muth et al. (*67*) tested the ability of bilberry anthocyanins to exert a positive role on night vision using young males with good vision. After three weeks of either receiving 160 mg of bilberry extract (25% anthocyanins) or placebo, the groups switched diets, and were subsequently tested on their night visual acuity and night contrast sensitivity. The data showed no significant difference in night visual acuity ($p>0.15$) and night contrast sensitivity ($p>0.35$) during active and placebo treatments. Therefore, the study failed to show any effect of bilberry on night visual acuity or night contrast sensitivity. Similarly, Levy and Glovinsky (*69*) also did not find any significant positive effects during the first 24 hr in subjects who received either an oral dose of 12, 24 or 36 mg anthocyanins (containing 12 mg anthocyanins as blueberry in addition to 2 mg beta-carotene). In a recent randomized double-blind placebo-controlled study, Lee and others (*37*) investigated the effect of purified high-dose anthocyanin oligomer administration on nocturnal visual function and clinical systems in low to moderate myopia subjects. There was a significant improvement in the anthocyanin group (73.3% improved symptoms) compared to the placebo group ($p<0.0001$). The anthocyanin group showed improved contrast sensitivity levels compared to the placebo group ($p<0.05$). The differences in the results between the studies could be due to the differences in the dose of anthocyanins given. Both Muth et (*67*) and Levy and Glovinsky (*69*) only used a tablet which contained about 25% anthocyanins, whereas Lee et al. (*37*) used a purified high-dose of anthocyanin oligomer (85%) which is significantly higher. Therefore, the observed improved effects on night vision are likely due to the increased bioavailability of the anthocyanin oligomer.

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The role of anthocyanins on vision has also been demonstrated in a few animal studies. The distribution of anthocyanins in tissues such as the liver, eye and brain tissue was investigated using a pig model by Kalt et al. (*35*). The results suggest that anthocyanins can accumulate in tissues, even beyond the blood-brain barrier. The highest amount of anthocyanins was found in the eye tissue with maximum concentration of 700 pg of anthocyanins/g of fresh weight. In another animal study, Matsumoto et al. (*36*) investigated the ocular absorption, distribution and elimination of blackcurrant anthocyanins in rats after oral and intraperitoneal administration and in rabbits after intravenous administration. The results demonstrate that blackcurrant anthocyanins are absorbed and distributed in ocular tissues and pass through the blood-aqueous barriers and blood-retinal barriers in both rats and rabbits.

Antidiabetic Properties

It is well known that dietary antioxidants including anthocyanins protect pancreatic beta cells from glucose-induced oxidative stress (*70*). Jayaprakasam et al. (*51*) investigated the glucose-induced insulin release from pancreatic beta-cells by anthocyanins and anthocyanidins *in vitro*, particularly the cyanidin, delphinidin and pelargonidn glycosides. Their results suggest that both anthocyanins and anthocyanidins are insulin secretagogues (enhance secretion). The most potent was delphinidin-3-glucoside which significantly induced the insulin secretion at 4 and 10 mmol/L glucose concentrations as compared to the untreated cells. Cyanidin-3-glucoside, however, was more active at lower concentrations, whereas pelargonidin-3-galactoside was the lowest insulin secretor. The results indicate that the number of hydroxyl groups in ring B of anthocyanins play an important role in their ability to secrete insulin. Zhang and others (*53*) also reported that several compounds present in grape skin or whole grapes are capable of enhancing insulin secretion as well as selectively inhibiting cyclooxygenase-2 enzyme.

A few *in vitro* and animal studies also investigated the role of anthocaynins in non-insulin-dependant diabetes mellitus. The retardation of alpha-glucosidase (AGH), a membrane-bound enzyme which catalyzes the cleavage of glucose from disaccharides, may be one of the most effective approaches to controlling non-insulin-dependant diabetes mellitus. An *in vitro* study by Matsui et al. (*71*) demonstrated that anthocyanin-containing plant extracts from *Clitoria ternatea* flowers and *Ipomoea batatas* purple sweet potato effectively inhibited AGH activity, suppressing the increase in postprandial glucose level. Matsui *et al.* (*30*) also demonstrated in rats that the anthocyanin extract from purple sweet potato is an effective AGH inhibitor as well as a potent antioxidant. These findings indicate a substantial benefit to diabetes patients which would be obtained by developing and consumption of a functional food containing anthocyanin extracts.

Tsuda and others (*72*) found that anthocyanins (cyanidin-3-glucoside) from purple corn (2 g/kg diet) significantly suppressed the development of obesity and decreased hyperglycemia induced by a high fat diet in mice. Guo et al. (*48*) also demonstrated that anthocyanin-rich extract from black rice is able to eliminate the glucose intolerance and hyperlipidemia initiated by 4 weeks of a high-fructose diet fed to rats. Rats fed diets supplemented with the black rice

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(5 g/kg of diet) exhibited lower oxidative stress than the fructose-fed controls, indicated by there lower concentrations of plasma TBARS and blood oxidized glutathione. Postprandial hyperglycemic events in diabetic patients are closely associated with increased oxidative stress and is one of the most important factors in the onset and progression of vascular complications in both type-1 and type-2 diabetes mellitus. In general, studies suggest that anthocyanins, as a functional food or supplement, can aid in the prevention of obesity and diabetes.

Anticancer Effects

In vitro studies have suggested that the anticancer activity of anthocyanins may be due to their anti-proliferative, pro-oxidant and apoptotic effects as well as their capability to regulate gene expression (*73*). Liu and others (*74*) demonstrated that the proliferation of human hepatocellular liver carcinoma is inhibited in a dose-dependant manner by raspberries. Fimognari et al. (*44*) identified cyanidin-3-glucoside as an anthocyanin compound capable of inducing apoptosis and cytodifferentiation in different leukemic cell lines. Treatment with cyanidin-3-glucoside was also found to revert human melanoma cells from the proliferating to the differentiated state (*75*).

Only limited data on anticancer effects of anthocyanins in animal and human is available. In an animal study, Hagiwara et al. (*46*) investigated the potential of purple corn to modify colorectal carcinogenesis in male rats supplemented for 36 weeks. These rats receiving the supplemented purple corn (21.5% anthocyanins, contributing to 5% of their diet), significantly reduced the incidence ($p<0.01$) and multiplicity of colorectal adenomas and carcinomas induced by 1,2-dimethylhydrazine (DMH) and 2-amino-1-methyl-6-phenylimidazo (4,5-beta) pyridine (PhIP). Kang and others (*45*) found that mice received anthocyanins supplemented diets significantly reduced caecal adenomas compared with those fed control diet or those that received the non-steroidal anti-inflammatory drug sulindac. Rats which received anthocyanin-rich extracts of chokeberry, bilberry or grape at 4 g/kg diet (approx 35 mg/animal/day) for one week before the carcinogen (azoxymethane-induced colonic aberrant crypt foci) had significantly reduced number of aberrant crypt foci compared to the control rats (*76*). The reduction was also accompanied with inhibition of cyclooxygenase-2-gene expression in malignant tissue. Similarly, rats who consumed approximately 0.38, 0.75 or 1.5 g black raspberries/animal/day decreased the multiplicity of azoxymethane-induced aberrant crypt foci by 21-36% and adenocarcinomas by 28-80% (*77*). Anthocyanins have also been shown to prevent skin cancer in rodents (*78*).

Bioavailability

Bioavailability is defined as the proportion of the nutrient that is digested, absorbed and metabolised through normal pathways (*79*). The majority of studies show that absorption of anthocyanins occurs quite rapidly following consumption (approx. 0.25-2 hr) and its excretion is complete within 6-8 hr (*80–86*). Results from various *in vitro*, animal and human studies all consistently

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show that anthocyanins are absorbed in their intact glycosidic form, contrary to other flavonoids (*80*, *87*, *88*). However, evidence shows that only less than 1% of the consumed anthocyanins are detectable in the plasma and urine (*81*, *82*, *85*). For more information about bioavailability of anthocyanins see our recent review (*55*). In general, studies have demonstrated that differences in anthocyanin structure and characteristics influence their antioxidant properties, bioavailability and overall health effects.

Food and Nonfood Applications

Food and nonfood additives including colorants have been used for centuries by industry to enhance or restore original appearance of products or to ensure uniformity as indicator of quality. Thus color is a major quality issue and manufacturers will do their best to retain the natural appearance of the raw material. However, during processing and storage, food color may be altered through the action of light, temperature, oxygen, metal ions or endogenous enzymes. Nowadays, more consumers are health-conscience and becoming increasingly more concerned about what they are eating and as a result, synthetic pigments are becoming increasingly rejected by the consumer. The acceptance of natural alternatives such as anthocyanin-rich fractions or pigments has been promoted due to their increasing health benefits. Extensive research on the health benefits of anthocyanins has drawn the attention to their use as functional food ingredients, natural food colorants, natural antioxidants and/or nutraceuticals.

Natural Colorants

A promising food color source should have a good hygienic and safety status, high color stability and purity along with a faint flavor and taste. In addition availability and economical-feasibility of anthocyanin-pigmented grains and milling and fractionation processes are also essential for sustainable development. A major drawback of anthocyanins as colorants is their decrease in tinctorial strength in low acid media. However, glucosylation and acylation was found to greatly improve the stability of the anthocyanin to pH changes, heat treatment and light exposure (*6*). Vegetable sources such as radish/purple sweet potato, black carrot, red-fleshed potato or red cabbage have been shown to provide a higher percentage of acylated anthocyanins than fruits which reflect a higher tinctorial strength of the respective extracts at food pH (*6*). Of these, radishes and red potatoes in particular have the potential to be used as an alternative for Federal Food Drug and Cosmetic Red No. 40 (Allura red). The color measurement showed that acylated pelargonidin derivatives extracted from red radishes imparted a red color to maraschino cherries close to that of Allura red at pH 3.5. Conversely, when anthocyanins are 5-glucosylated such as in red cabbage, they tend to lose their color more easily, which is characterized by their hydration constants, with higher values corresponding to higher coloring efficiencies at a given pH (*89*). Thus the chemical structure of anthocyanins is influential in

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the development of anthocyanins as natural colorants due to its impact on color stability and shelf life.

Red rice is commonly used as a food colorant in China (*22*). Red rice is approved by the Chinese Ministry of Health as a modern food additive to increase the color and delicacy of meat, fish and soybean products as part of the Chinese diet (*90*). Successful food applications have also been reported for red cabbage and radish extracts, made only possible after the development of suitable procedures to remove or drastically reduce the concentration of aroma and flavour compounds (*91*). Other applications of anthocyanin extracts include coloration of acid fruit preparations, jams and preserves. However, the use of extracts in these applications is highly dependable on the nature and quality of the fruit (fresh, frozen or sulphited) and whether proteins are present. For example, extracts containing other phenolics or oliogomeric pigments above a certain level cannot be used to color jellies as they form precipitates with gelatine (*92*). In confectionary sugar, grape extract (0.4% w/w) produces a clear ruby red color in boiled sweets, which can be adjusted with colorants from other fruit sources. Giusti and Wrolstad (*91*) investigated the viability of acylated anthocyanins from red radish, red cabbage, black carrot and grape skin extract to color dairy products such as yogurt and sour cream having pH levels around 4.2-4.5. They discovered that radish and carrot alone or in combination could provide a desirable red hue for dairy applications at concentrations as low as 5 mg monomeric anthocyanin/100 g sample. Since the shelf-life of these tested dairy products are only a few weeks under refrigeration, the stability of anthocyanin extracts would not be affected making them viable alternatives. With increasing pH value, discoloration occurs, but if the product being colored contains components capable of acting as co-pigments, color may be retained and also light-stabilised to a certain extent (*92*). Therefore, the use of acylated anthocyanins with improved color and stability to heat, light and pH could hold a promise for incorporating anthocyanins as natural food colors. It is predicated that in the future, the production and adding of anthocyanins as natural food colorants will steadily increase, following the current trend away from synthetic colors (*10*). Purple corn has been used as a food colorant since 1977 (*23*). In addition, blue and purple corn is processed into naturally colored snack foods such as tortilla.

Regulatory policies concerning the use of anthocyanin-based colorants differ considerably from country to country. Many countries limit the approval of colorant usage to defined food products, and do not permit their use in such commodities as milk and fruit juice. The European Union, Australia, Canada, Cyprus, Finland, Japan, New Zealand, Norway, South Africa, Sweden, Switzerland, and the USA restrict anthocyanin usage to defined food products (*93*). Countries that do have general approval for food use of anthocyanin colorants are Chile, Columbia, Iran, Israel, South Korea, Malta, Peru, Saudi Arabia and the United Arab Emirates. In the European Union, all anthocyanin-derived colorants are recognized as natural colorants under classification E 13. In the U.S., 4 of the 26 colorants that are exempt from certification and approved for food use are anthocyanin-derived: grape skin extract, grape color extract, fruit juice, and vegetable juice (*93*). In addition to grape, the more common fruit and vegetable sources include red cabbage, blackcurrants, radishes, elderberries, chokeberries,

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blackberries, black raspberries, and black currants. Blue wheat could also be a promising source for the production of anthocyanin colorants (*33*).

Functional Food Ingredients

Berries and black currants are rich sources of anthocyanins, but purple and blue pigmented grains also contain high amounts of anthocyanins. Grains can also be fractionated by dry milling processes to produce milling fractions that are very rich in anthocyanins such as black rice bran (25354 μg/g) and blue wheat bran (818 μg/g) (*12*). Due to the widespread occurrence of anthocyanins in fruits, vegetables and grains a high amount is consumed by humans. Depending on the country and nutritional habits of the individuals, the daily intake of anthocyanins has been estimated to range from several milligrams to hundreds of milligrams per person (*10*). In USA the dialy intake of anthocyanins is estimated at 12.5 mg/day (*4*). Intake of anthocyanins is steadily increasing because extracts and juices from fruits and vegetables with high anthocyanin contents are becoming much more commercially available nowadays, and health benefits of anthocyanins have become well evident.

Anthocyanin applications in food systems are preferably used in acidic food to assure a predominance of the flavylium cation. For example, blue wheat anthocyanins, either in whole meal or isolated form are most thermally stable at pH 1, and their degradation is insignificantly lower at pH 2 as compared to pH 5 (*27*). This could explain the main use of anthocyanin extracts such as grape pigments in beverages and soft drinks approximately 3 kg of a 1% anthocyanin extract added to 1000 L of beverage can impart a deep red color (*92*). Grape extracts have also been shown to be resistant to sulphur dioxide (SO_2) bleaching [109]. Addition of SO_2 is widely used as a preservative in beverages which may cause anthocyanin color to be bleached initially, but then partially restored as SO_2 becomes oxidised (*92*). It has also been demonstrated that the addition of SO_2 during heating of blue wheat increased stabilization of the anthocyanin pigments compared to the control sample or heating without SO_2 (*27*).

The majority of blue, purple or red corn is currently used for ornamentation due to its colorful appearance with only a small amount being utilized in the production of blue and pink tortillas. Purple wheat is crushed into large pieces, which are spread over the exterior of multigrain bread (*94*). Abdel-Aal et al. (*12*) suggested that blue, pink and purple corn which possess relatively high amounts of anthocyanins, especially purple corn (965 *u*g/g) (Table 3) holding a promise for the development of functional foods and/or natural colorants. Similar to other bioactive compounds, the environment in which they are grown was determined to have a significant influence on their anthocyanin concentration and composition. Therefore, during crop production efforts should be made to ensure maximum anthocyanin content for both fruits and cereals. Black sorghum has also been shown to contain significant levels of anthocyanins and other phenols concentrated in the bran fraction being significantly higher than other sorghums (*31*). The bran fraction was found to contain approximately 4.0-9.8 mg/g of anthocyanins mainly 3-deoxyanthocyanidins such as luteolinidin and apigeninidin. This amount is relatively high compared to pigmented fruits and

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vegetables (0.2-10 mg/g) on a fresh weight basis. Therefore, black sorghum has a good potential as a functional food product.

Dietary Supplements

Anthocyanin extracts from purple corn have been incorporated as an antioxidant dietary supplement with claims to promote healthier, younger looking, more radiant beautiful skin (*95*). Anthocyanins are also being sold as a supplement called Medox which incorporates a concentrated amount of cyanidin-3 glucoside and delphinidin-3-glucoside extracted from Norweigan bilberries (*Vaccinium myrtillus*) and black currants (*Ribes nigrum*) (*96*). Red rice is also being fermented and sold as a dietary supplement and marketed as Cholestin to help reduce cholesterol levels (*97*). Additionally, blue wheat bran can be further processed into anthocyanin-rich powder as a dietary supplement (*33*). In general, anthocyanins are gradually being incorporated into the food and beverage products as a food colorant, functional food or dietary supplement. Increased development of anthocyanins with enhanced stability and prolonged shelf life will increase their food applications and overall consumption and thereby increase its positive role in human health.

Conclusions

Many *in vitro* and *in vivo* studies have shown the significance role of anthocyanins in human health and nutrition. In addition the widespread occurrence of anthocyanins in our diet has also provided significant evidence for the development of anthocyanins as a functional food and/or dietary supplement. Grains as staple foods would offer a huge vehicle for the enhancement of anthocyanins intake through the development of wholegrain foods made from anthocyanin-pigmented grains such as blue and purple corn, black sorghum, blue and purple wheat, and black and red rice. Rapid advancements in the food technology and analysis have allowed for efficient extraction, processing and identification of anthocyanin compounds from various agricultural commodities to be increasingly incorporated into the food and beverage industry as natural colorants, functional foods and dietary supplements. Since it is important to understand the nature of absorption and metabolism of anthocyanins *in vivo*, as well as the impact of processing on bioactivity of these compounds, more research is required to address these issues. At present relatively little is known about absorption of anthocyanin compounds following consumption and of the mechanisms by which they exert their beneficial health effects.

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Chapter 6

Autohydrolytic Production of Feruloylated Arabinoxylan Hydrolysates from Cereal Processing Coproducts for Food Applications

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Cereal brans are rich sources of dietary fiber and phenolic antioxidants. The chief portion of dietary fiber in cereal brans is insoluble arabinoxylans, which are cross-linked via ferulate bridges and poorly fermentable by gut bacteria. Because many functional properties and health benefits of dietary fiber rely on solubility and microbial fermentation, it is desirable to develop strategies to solubilize the arabinoxylans in cereal brans. Autohydrolysis describes the process of subjecting cereal brans to high temperatures (160-220 °C) in the presence of water, releasing a portion of the insoluble arabinoxylans in the form of feruloylated arabinoxylan hydrolysates. These hydrolysates may impart several health benefits that deserve exploration.

Introduction

The US diet is woefully deficient in dietary fiber: the average citizen consumes about half of the daily recommendation (*1*, *2*). The consequences of consuming a diet low in dietary fiber cannot be ignored; low-fiber diets have been associated with obesity, cardiovascular disease, type 2 diabetes, and certain types of cancer (*3–19*). Interestingly, cereal dietary fibers have shown stronger correlations with disease resistance than dietary fibers from other sources (*5*, *11*, *13*, *16*, *20*, *21*).

In the past, governments have responded to widespread nutrient deficiencies by fortifying staple foods with the deficient nutrient (e.g., flour fortification, iodization of salt). Unfortunately this strategy is not a viable option for delivery of

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dietary fiber. Dietary fiber, unlike vitamins or minerals, must be added to foods in gram quantities per serving in order to be of dietary significance. Not surprisingly, this can have a profound effect on the organoleptic properties of the food through electrostatic interactions, hydrogen bonding, and non-polar interactions with other food components (*22*). Dietary fiber also encompasses a broad range of compounds with widely different chemical and physical properties. These dietary fibers are often used in foods in highly impure forms, which contain a number of compounds that are not dietary fiber, but may influence texture, flavor, color, and nutritional value (*23*).

Creating dietary fiber ingredients that promote optimum health and contribute minimal impact on food organoleptic properties creates a unique and challenging opportunity for food and carbohydrate chemists. The purpose of this chapter is to discuss the properties, production, and potential health benefits of feruloylated arabinoxylan hydrolysates (FAH), especially those produced by autohydrolysis of cereal processing coproducts for food applications.

Structure

Parent Polysaccharide: Arabinoxylan

Hemicelluloses are important cell wall structural components of plants. These polysaccharides include xylans, galactans, mannans, and non-cellulosic β-glucans. The focus of this chapter will be on xylans, specifically arabinoxylans, which are the most abundant hemicellulosic components of cereal grains. Arabinoxylans are found in all anatomic sections of grains, but are concentrated in the pericarp, or bran (Table I). Arabinoxylans in cereals consist of a backbone of 1, 4-linked β-D-xylopyranosyl units. The backbone is substituted with, most commonly, single α-L-arabinofuranosyl units at C-(*O*)-2 and C-(*O*)-3. Other substituents on the backbone include acetate, glucuronic acid, 4-*O*-methylglucuronic acid, and complex oligomeric side chains consisting of arabinose, galactose, glucose, uronic acids, and xylose (*24*). One particularly unique feature of arabinoxylans is that some of the arabinose side groups are substituted with hydroxycinnamic acids, including ferulic (and oligomers of ferulic), p-coumaric, and sinapic acids (*41*). The types, distributions, and quantities of substituent groups on the xylan backbone vary within cereals depending on location within the grain (*42*) and, more substantially, among different cereals (*24*, *43*). These differences in structure not only contribute important structural features to the cell walls of grains, but also distinct functional (*44*, *45*) and nutritional properties (*38*, *46*).

Feruloylated Arabinoxylan Hydrolysates

Arabinoxylans in cereals can be water-soluble or -insoluble depending on covalent and non-covalent interactions among arabinoxylan chains and among other grain components. In all cereal grains, the majority of arabinoxylan is insoluble (Table I) and thus is often difficult to incorporate into foods without changing product quality and acceptability. Furthermore, literature suggests that many of the disease prevention characteristics of dietary fibers arise from their

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metabolism by bacteria in the gut (*47*, *48*); however, native arabinoxylan is poorly fermented by these bacteria (*38*). Partial hydrolysis of insoluble arabinoxylan creates water-soluble oligosaccharides with low viscosity that are metabolized by gut bacteria and can contribute potentially beneficial bioactivity (*49*). These characteristics are desirable from both food processor and nutritional points of view. Therefore, there is tremendous interest in developing strategies to produce FAH from cereal processing coproducts.

Table I. Arabinoxylan content of various cereals (*24–40*)[a]

Grain	*Total*[b]	*Water-extractable*	*Water-unextractable*[c]
Wheat			
Wholemeal	5.8–7.6	0.42–0.99	5.4–6.6
	(0.60–1.00)		
Bran	12.7–22	0.30–0.85	12–21
	(0.55–0.70)	(0.70–1.65)	
Rye			
Wholemeal	5.6–9.8	1.3–2.5	4.3–7.3
	(0.55–0.57)	(0.64)	
Bran	12-15	1.0–1.2	11–14
	(0.48–0.58)	(0.72–0.78)	
Barley			
Wholemeal	3.7–16	0.36–3.1	3.3–13
	(0.35–0.60)	(0.25–1.10)	
Bran	4.8–9.8	0.15–0.35	4.7–9.5
	(0.54–0.67)	(0.80–1.1)	
Corn[d]			
Bran	43–59	—[e]	43–59
	(0.54–0.58)	—	

[a] Arabinoxylan content as reported in the citation or as the sum of xylosyl and arabinosyl residues in the product less the arabinose contributed by arabinogalactan (0.69 arabinose:galactose ratio) [b] Expressed as % dry matter; arabinose:xylose ratio reported parenthetically [c] Calculated by difference (total less water-extractable) [d] Heteroxylan content reported rather than arabinoxylan; expressed as the sum of arabinosyl, xylosyl, galactosyl, and glucuronosyl residues [e] Not present

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In the literature, hydrolysates of arabinoxylan are referred to as arabinoxylan oligosaccharides (*49*), feruloylated arabinoxylooligosaccharides (*50*), arabinoxylooligosaccharides (*51*), feruloyl xylooligosaccharides (*52*), xylooligosaccharides (*53*), cinnamoyl-oligosaccharides (*54*), and feruloylated oligosaccharides (*55*), often without much consideration as to whether the term exactly describes the chemical structure of the product under study. For the purposes of this chapter, the term FAH will be used to describe hydrolysates of arabinoxylan with degrees of polymerization (DP) ≥ 2 that have not been treated in such a way that would be expected to remove the ferulic acid (e.g., alkali, feruloyl esterase, arabinofuranosidase; Figure 1).

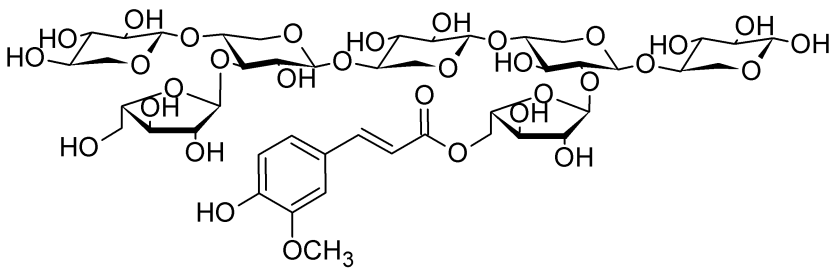

Figure 1. One of the many possible structures of feruloylated arabinoxylan hydrolysates. The structure consists of a (1, 4)-linked β-D-xylopyranosyl backbone with 2 to >1000 degrees of polymerization with α-L-arabinofuranosyl side groups at positions C-(O)-2 and/or C-(O)-3, some of which contain the antioxidant ferulic acid esterified at C-(O)-5. Uronic acids, oligomeric side groups, acetic acid, and hydroxycinnamic acids other than ferulic acid may also be present.

FAH retain many characteristics of the parent polysaccharide from which they came: they possess wide variation in molecular weight, branching, and presence of ferulic acid, which may be important with respect to production parameters and health benefits.

Occurrence of FAH

FAH are present in most cereal-based foods (*56*), albeit at relatively low concentrations. During food processing, endogenous or added enzymes can act on native soluble and insoluble arabinoxylan and produce these products. Among the most substantial sources of natural FAH are beer (*57*) and bread (*58*). Recently Dorenz et al. (*59*) described a method for increasing FAH in bread by adding a high level of a xylanase that is active during the early stages of baking.

Although not commercially available on a large scale, much research has been devoted to producing FAH that could be added to food as a health-promoting ingredient (*56*, *60*). In this approach, FAH could be found ubiquitously in foods at dietarily-significant levels.

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Production of FAH as Food Ingredients

Many strategies for producing FAH have been described. These methodologies can be divided into two main categories: those produced by enzyme treatment, and those produced by autohydrolysis (*56*, *60–62*). In the context of this chapter, both techniques will be described, but special emphasis on the unique characteristics of FAH from autohydrolysis will be highlighted. Furthermore, although the production of FAH and similar type products from many different agricultural products have been described, the focus of this chapter will be on production from cereal-based coproducts such as bran, hulls, and other materials with emphasis on coproducts arising from food-grade operations for eventual use as food ingredients.

Enzyme

Enzymatic production of FAH, which is the most common means of producing FAH, involves treating the material containing arabinoxylan, or the isolated polysaccharide itself, with enzyme(s) that hydrolyze portions of the arabinoxylan (Figure 2). Endo-(1,4)-β-xylanases (xylanases; EC 3.2.1.8), the principle enzymes used in this process, cleave the (1,4) glycosidic linkages between β-D-xylopyranosyl residues on the backbone. Xylanases are classified into glycoside hydrolase (GH) families based on amino acid sequence similarities (http://afmb.cnrs-mrs.fr/CAZY/). Xylanases have been identified in GH families 5, 7, 8, 10, 11, and 43; although only xylanases from families 10 and 11 are active on arabinoxylans (*63*). In general, GH10 xylanases are less impeded by substituted regions of the arabinoxylan chain than GH11 and are capable of releasing xylose as a main end product; GH11 xylanases usually show more activity on high molecular weight arabinoxylans and release oligosaccharides as their main end product (*63*). For creation of FAH it is desirable to identify xylanases that are most active on insoluble arabinoxylan, referred to as substrate selectivity (*64*). Moers et al. (*64*) tested six different xylanases for substrate selectivity and found that a GH11 xylanase from *Bacillus subtilus* was the most selective for insoluble arabinoxylan. Unfortunately, GH family alone cannot predict a xylanase's propensity for hydrolyzing insoluble substrate, as enzymes from the same GH family exhibit wide variations in substrate selectivity (*64*, *65*).

When producing FAH with xylanases, not all of the insoluble arabinoxylan is released from its insoluble matrix. This is due to the complex and highly substituted nature of arabinoxylans. Yield of FAH from insoluble arabinoxylan from cereal grain coproducts generally ranges from 9-45% (Figure 3). Yield of oligosaccharides can be enhanced by adding other enzymes such as arabinofuranosidases or feruloyl esterases (*74*, *75*), although these will also remove ferulic acid from the FAH.

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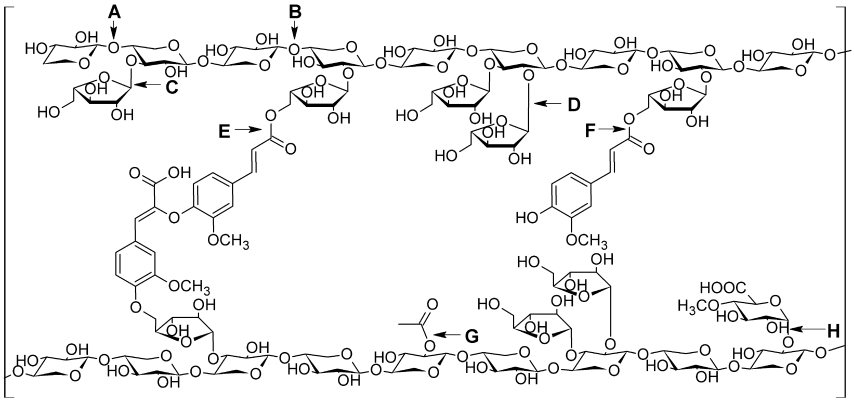

Figure 2. Sites of hydrolysis for various enzymes that are active on arabinoxylans: A) exo-xylanases (xylosidases); B) endo-xylanases; C, D) α-L-arabinofuranosidases (arabinases); E, F) feruloyl esterases; G) acetyl esterases; H) α-glucuronidases.

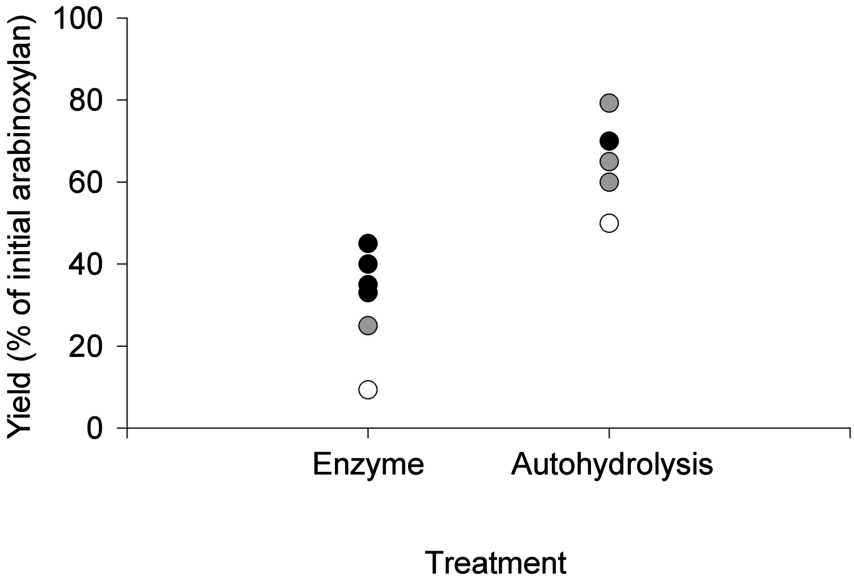

Figure 3. Yield of polymeric arabinoxylan hydrolysates from corn bran (white circles), corn cobs (gray circles), and wheat bran (black circles) after enzyme (xylanase) and autohydrolysis treatments (50, 54, 66–73).

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Autohydrolysis

Autohydrolysis of arabinoxylan occurs when arabinoxylan is subjected to hydrothermal processing (160-220 °C). At high temperatures, water dissociates and substituent acids present on the native arabinoxylan, principally acetic acid, are liberated and catalyze acid hydrolysis of the polymer, releasing soluble hydrolysates of arabinoxylan while leaving cellulose and lignin largely unmodified (*61*, *62*). In contrast to the selective nature of enzymatic processing, autohydrolysis results in the production or release of a number of undesirable non-saccharide contaminants, including acetic acid, lignin degradation products, furfural from pentose dehydration, soluble inorganic compounds, and protein fragments. Moreover, autohydrolysis generally results in much higher release of monosaccharides compared with enzyme processing. The release of these compounds can be minimized by employing a two step reaction scheme (*62*). The first step is performed at a temperature that does not induce autohydrolysis. Soluble materials are then washed away and the second autohydrolysis step is performed to release the FAH. This reduces but does not eliminate non-saccharide components in autohydrolysate liquors. Following autohydrolysis, non-saccharide components can be removed by solvent extraction, activated carbon, or ion-exchange; monosaccharides can be removed by ultrafiltration or chromatography (*62*, *68*, *76–78*). Unfortunately all of these purification strategies introduce significant production costs.

Comparison of Enzyme and Autohydrolysis

The costs associated with producing FAH either with enzyme or autohydrolysis have been restrictive. More interest has been shown in enzymatic production of FAH due to the selective nature of xylanases—fewer non-saccharide components are released during treatment—and the procedure does not require expensive capital equipment that is capable of withstanding the pressure needed to induce autohydrolysis (*60*). Nevertheless, only one commercial product produced by enzymatic treatment of arabinoxylan from corn cobs is available in Japan (Xylooligo, Suntory Ltd.). This product is technically not an FAH, but is similar: it contains xylobiose and xylotriose without arabinosyl side groups or ferulic acid as the principle components.

While enzymatic treatment may appear to be far better than autohydrolysis for FAH production, autohydrolysis does possess some advantages. For instance, conversion of insoluble arabinoxylan to soluble oligosaccharides (DP $\geq$ 2) upon xylanase treatment ranges from 9-45% (as a percent of initial arabinoxylan content), depending on choice of enzyme, starting material, and reaction conditions, whereas autohydrolysis results in 50-80% yields (Figure 3).

A second and perhaps more important advantage is that autohydrolysis may be used to produce FAH from starting materials on which most available xylanases exhibit limited activity. Limited activity of xylanases on some agricultural products is due to complexity in branching pattern and the presence of ferulic acid cross-links. Corn bran for instance possesses a particularly branched and cross-linked arabinoxylan often referred to as a heteroxylan due to its complex

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nature (*25*, *79*). Indeed, corn heteroxylan contains much more branched arabinose and terminal xylose units (components of multi-unit, complex branches) and more than 7 times the di- and tri-ferulate (cross-links) than other common cereal grains (*79–83*). Figure 3 shows a 9% yield of arabinoxylan oligosaccharides from corn bran by enzyme treatment. In this study (*73*), the authors subjected the corn bran to an autoclave (121 °C, 60 min) treatment prior to xylanase hydrolysis to enhance enzymatic access to the polymer; xylanases from both GH families 10 and 11 show nearly no activity on arabinoxylan from corn bran without any pretreatment (Figure 4). Using autohydrolysis, however, FAH were produced from corn bran in 50% yields (with an autoclave pretreatment at 121 °C for 45 min; (*72*)). FAH with such complex structure as those derived from corn bran may be desirable due to the potential for protracted fermentation in the large bowel and presence of higher levels of antioxidants (see Potential health benefits of FAH produced by autohydrolysis).

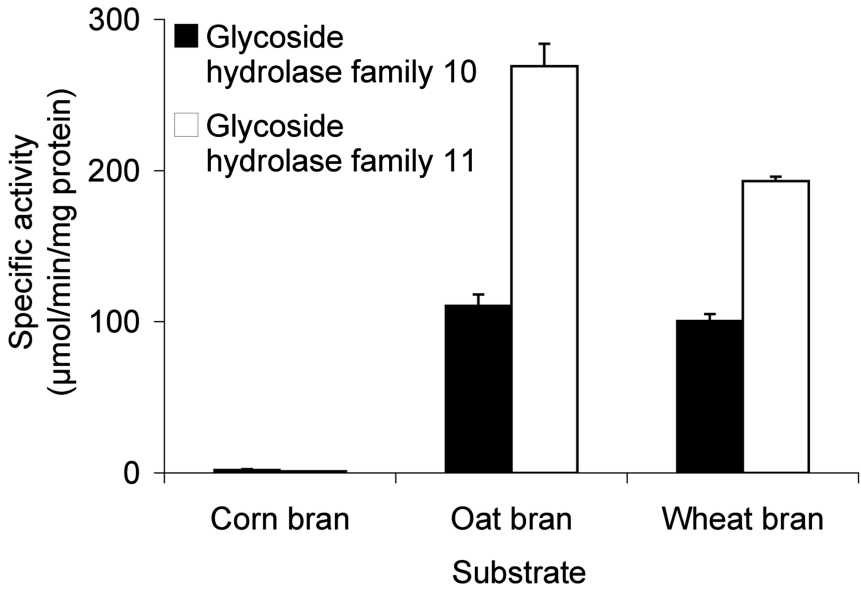

Figure 4. Activity of two xylanases on insoluble arabinoxylan from three cereal brans (84).

As mentioned, FAH retain many structural characteristics of the parent polysaccharide from which they came, regardless of whether they are produced by enzyme or autohydrolysis. However, molecular weight profiles of FAH from enzyme or autohydrolysis treatment are quite different. Whereas enzymatic hydrolysis results in polymers that fall into the traditional oligosaccharide range, i.e., DP 2-20 (*51*, *69*), hydrothermal processing results in a mixture of both oligo- and polysaccharides (DP > 20; Figure 5). Differences in molecular weight profiles may play a role in the health benefits of FAH (see Potential health benefits of FAH produced by autohydrolysis).

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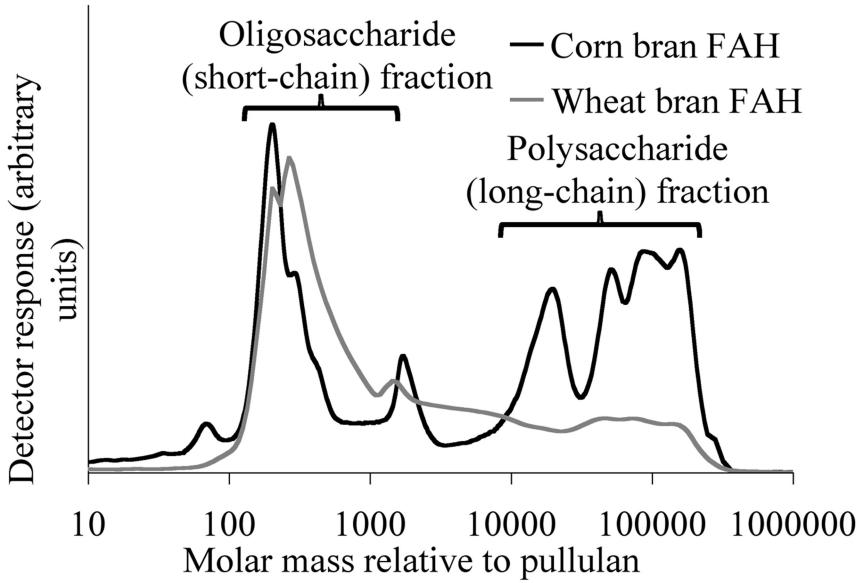

Figure 5. Molar mass distribution of two feruloylated arabinoxylan hydrolysates (50, 72). Corn bran curve reprinted with permission from reference (50). Copyright 2010 Elsevier.

Potential Health Benefits of FAH Produced by Autohydrolysis

The health benefits of dietary fiber were once thought to be confined to fecal bulk and laxation (*85*). Today it is recognized that dietary fiber plays key roles in the prevention of many diet-related diseases, including heart disease, diabetes, obesity, and certain gastrointestinal disorders (*86*). Few studies have addressed the particular health promoting effects of FAH produced by autohydrolysis; however, these products have a number of potential health benefits that can be surmised from literature on arabinoxylan oligosaccharides produced by enzyme treatment, from long-chain arabinoxylan isolated by alkali, and from other health-promoting dietary ingredients. Figure 5 shows that FAH contain both oligo- and polysaccharide fractions. These are accompanied by ferulic acid esterified to the polymers. In this section, each of these components will be addressed in the context of potential health benefits that may be derived.

Oligosaccharide Fraction

Dietary fiber fermentation by bacteria in the gut and its influence on the microbiota composition and function is recognized to be of profound importance (*47*, *86*). The gut microbiota plays roles in gut tissue development, energy balance, nutrient absorption, detoxification, disease resistance, and immune system development and regulation (*87*, *88*). Thus, these bacteria have the ability

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to affect the development of many diseases that plague modern society, including obesity, diabetes, heart disease, and some types of cancer (*87*, *89–92*).

Dietary fibers that provoke beneficial shifts in the gut microbiota and thus benefit the host by precluding growth of detrimental bacteria are referred to as prebiotics (*47*). Beneficial bacteria reduce the growth of detrimental bacteria through competitive inhibition, production of antibacterial compounds, and production of short chain fatty acids (SCFA; (*93*)), which are products of anaerobic carbohydrate fermentation. SCFA also acidify the colonic environment, which favors mucin production and reduces the activity of co-carcinogenic enzymes (*94*). The SFCA butyrate in particular has trophic effects on the host, serving as an energy source for colonic enterocytes, a signaling molecule for the mucosal immune system, and a factor that stimulates production of protective mucins that separate the epithelium from the microbiota (*94*, *95*). Prebiotics themselves are also hypothesized to influence immune function by interacting with receptors on immune cells (*96*). The combined effects of prebiotics would likely be of benefit against multiple types of metabolic disorders associated with a low-grade inflammation, such as obesity and type 2 diabetes (*97*, *98*), or contribute to long-term prevention of chronic subclinical inflammation that can develop into inflammatory bowel disease and colorectal cancer (*99–101*).

Although new techniques for analyzing gut microbial communities are beginning to uncover new beneficial bacteria that could be targets for new prebiotics (*102*, *103*), the genera of bacteria most commonly targeted for gut health with prebiotics have been *Bifidobacterium* and *Lactobacillus*. These bacteria thrive on oligosaccharides and grow poorly on long-chain polysaccharides. For this reason, all current prebiotics are oligosaccharides (*104*, *105*).

Fructans, the most commercially successful and studied prebiotics, which include fructooligosaccharides (FOS), or oligofructose, inulin, and related products, have been shown to be particularly bifidogenic, leading to reduced production of potentially toxic metabolites and stimulation of important immune-mediated effects (*93*).

Compared with fructans, FAH and related oligosaccharides are still in their infancy with respect to research. Nevertheless, Aachary et al (*60*) and Broekaert et al (*56*) have recently described the prebiotic nature of oligosaccharides produced from arabinoxylan hydrolysis. These products are readily utilized by *Bifidobacteria* and result in beneficial shifts in mixed gut microbial communities (*106*, *107*). Some studies even suggest that the bifidogenic nature of arabinoxylan oligosaccharides may exceed that of fructans (*53*, *106*, *108*, *109*).

The selective bifidogenic nature of arabinoxylan oligosaccharides may be due to specific oligosaccharide uptake mechanisms for these compounds exhibited by some species (*110*). Interestingly, *Bifidobacteria* utilize arabinoxylan oligosaccharides through intracellular xylosidases and arabinofuranosidases; they do not express endo-xylanases (*111*, *112*). This means that they can efficiently metabolize arabinoxylan oligosaccharides, but cannot ferment unmodified or long-chain arabinoxylan unless it is partially broken down by other commensal bacteria that do express endo-xylanases, such as *Bacteroides* and *Roseburia* (*113*, *114*).

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Polysaccharide Fraction

Because currently accepted probiotic bacteria do not grow on polysaccharides, the importance of these carbohydrates on gut health is often overlooked. However, the gut microbiota survives in large part on the indigestible polysaccharides that are consumed by the host. Indeed, it is estimated that 32-80 g of fermentable carbohydrate are needed to sustain the energy requirements of the human gut microbiota (*115*, *116*). It is clear that on Westernized diets the quantity of indigestible carbohydrate supplied to the gut microbiota is grossly inadequate, generally falling short of even 20 g/d (*2*). This has led some to hypothesize that the current rise in diet-related diseases in the developed world is a result of sluggish bacterial metabolism in the colon as a result of an inadequate supply of fermentable carbohydrate (*117–119*). As a consequence, the composition of the gut microbiota shifts away from beneficial saccharolytic bacteria toward proteolytic and scavenging bacteria; organisms begin to forage for nutrient sources, oftentimes targeting breakdown of glycosylated proteins (mucin) which normally protect the epithelium, or breaking down protein into phenols and ammonia, setting the stage for a number of undesirable effects on the human host.

Were this the whole story, intake of fermentable carbohydrate in any form would be enough. However, conditions throughout the colon vary widely from the proximal to distal colon (Figure 6). The cecum receives indigestible carbohydrate from the terminal ileum; thus this region generally supports beneficial saccharolytic fermentation. Moving further into the colon, however, readily fermentable carbohydrate is exhausted and bacteria begin salvaging for nutrients, resulting in unfavorable conditions. This is likely why prevalence of disease increases when moving from the proximal to distal colon (Figure 6). Because fermentable carbohydrate is so important for maintaining a healthy gut environment, to harvest the full benefits of prebiotics and other dietary fibers they must be capable of supporting saccharolytic bacterial fermentation even after prolonged fermentation.

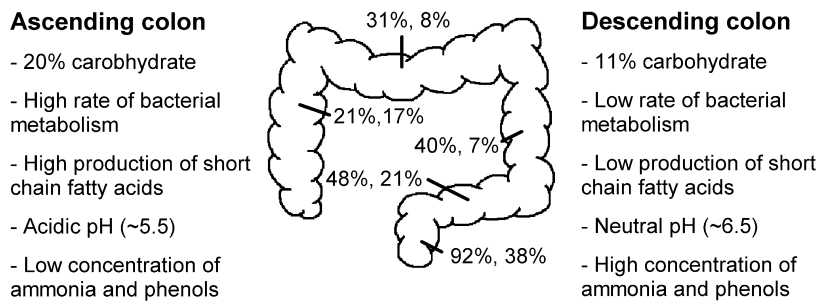

Figure 6. Differences between conditions in the ascending (proximal or right) colon and the descending (distal or left) colon (120, 121); numbers refer to prevalence of disease in each region (first number = ulcerative colitis; second number = cancer; (122, 123)).

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Generally, short chain oligosaccharides are utilized very rapidly by bacteria (*124*, *125*). This limits the benefits that can be derived from these products (*126*). With this in mind, a commercial 50:50 mixture of fructooligosaccharides and long-chain inulin has been developed (Raftilose Synergy 1, Orafti) and shown a number of benefits over FOS, including enhanced mineral absorption and inhibition of carcinogenesis (*127*, *128*). This has been attributed to a two phase fermentation that initially stimulates beneficial bacteria in the proximal portions of the colon (rapid fermentation of the oligosaccharide fraction), followed by a protracted fermentation of the polysaccharide fraction by the same groups of bacteria into the distal regions of the colon (*127*, *128*).

Because FAH contain both oligo- and polysaccharides, this same principle could possibly be applied to FAH produced by autohydrolysis. Thus, the short chain fraction would be expected to elicit a prebiotic response, while the polysaccharide fraction would support these beneficial bacteria in more distal regions of the colon. Notably, autohydrolysis can liberate portions of arabinoxylan that cannot be liberated by available xylanases (*72*, *84*). These products have very complex, branched structures, which may support even greater protracted fermentation than other polysaccharides (such as inulin), but need to be studied in more detail to substantiate this claim.

Feruloyl Moiety

FAH, whether produced by enzyme or autohydrolysis, have the unique structural component ferulic acid (Figure 1). Despite ferulic acid's antimicrobial activity against spoilage and pathogenic microorganisms (*129*, *130*), it does not appear to inhibit the growth of *Bifidobacteria* (*131*, *132*). On the contrary, ferulic acid may impart a number of substantial benefits. For instance, ferulic acid is a potent antioxidant *in vitro*; among other antioxidant phytochemicals, ferulic acid is the most inhibitory of lipid and protein oxidation in a lecithin/liposome oxidation system (*133*). Interestingly, FAH are even more potent free-radical scavengers than free ferulic acid (*134*). Of note, however, is that *in vitro* measurements of antioxidant activity have been under fire as not having relevance to *in vivo* conditions because they do not take into account bioavailability, metabolism, and other physiological factors (*135*). Moreover, the host organism (the human body) contains a number of pathways designed to prevent oxidative damage.

In vivo studies have suggested that, like other phenolic acids and plant secondary metabolites that are antioxidants, ferulic acid can confer benefits by influencing cell signaling and reducing the inflammatory cascade (*136*, *137*). For example, Yeh et al. (*138*) showed that ferulic acid up-regulated gene expression of cardiac antioxidant enzymes in rats.

Free ferulic acid is rapidly absorbed in the proximal gastrointestinal tract (*139*). Most dietary ferulic acid, however, is not found in its free form, but it bound to carbohydrates. In cereal products, most of the ferulic acid is bound to insoluble arabinoxylans and is only slightly bioavailable (*140*). However, when ferulic acid is esterified to soluble oligo- or polysaccharides, it is readily released by bacterial feruloyl esterases in the large intestine and becomes bioavailable (*141*).

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Because ferulic acid on FAH reaches the colon, it may also provide benefits to the colonic environment. The lumen of the colon is not protected by the body's natural free radical defense mechanisms; however, under some circumstances, such as during inflammation, this region can be bombarded by free radicals (e.g., nitric acid), which are produced by neutrophils, and result in mucosal oxidative stress, peroxidation of membrane lipids, and tissue damage (*142–145*). Dong et al. (*146*) showed that enemas of ferulic acid were effective at reducing inflammation during flare up of inflammatory bowel disease. Ferulic acid on FAH may provide similar benefit without having to be administered by enema.

Autohydrolysis may be used to produce FAH with very high levels of ferulic acid. As mentioned, enzyme treatment cannot be used solely to produce FAH from corn bran in sufficiently high yields. However, corn bran contains more than 7 times the ferulic acid than other cereal processing coproducts. Thus, this would be an ideal starting material for the production of FAH with very high levels of ferulic acid. Rose et al. (*72*) produced FAH from corn bran by autohydrolysis in 50% yield that contained 8.0 g of esterified ferulate/100g arabinoxylan hydrolysate. By comparison, Lequart et al. (*54*) produced FAH from wheat bran (the most common source) in 35% yield that contained 0.88 g esterified ferulate/100g arabinoxylan hydrolysate.

Conclusion

FAH are polymeric (DP ≥ 2) hydrolysates of arabinoxylan that contain ferulic acid as a structural component. These products can be produced by enzyme or autohydrolysis, which result in related products with some important differences. Most notably, autohydrolysis can be used to produce FAH from agricultural products on which available xylanases possess little activity and autohydrolysis releases both oligo- and polysaccharides while enzyme does not release polysaccharides. FAH produced by autohydrolysis possess a number of unexplored benefits that can be predicted based on analysis of similar products. In particular, FAH from autohydrolysis may serve as prebiotics through their oligosaccharide fraction and slowly fermentable dietary fibers through their polysaccharide fraction. This would create a healthier colonic environment especially in the distal colon, which is particularly disease-prone. Also, the ferulic acid moiety may impart substantial anti-inflammatory effects in the large intestine or exhibit systemic benefits through release by gut bacteria and subsequent absorption. Substantiating these potential benefits may help to create new functional ingredients that prevent or even treat disease. In light of the declining health status of individuals in developed countries this research is imperative.

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Chapter 7

Sorghum Protein Structure and Chemistry: Implications for Nutrition and Functionality

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Sorghum is the 5th most widely grown cereal crop in the world and has desirable agronomic traits such as drought resistance and heat tolerance. Sorghum is a major food source in developing nations and is widely used as feed grain in Western countries. There is increasing interest in sorghum food products for people with celiac disease in Western countries as well. Sorghum endosperm proteins are known to have equal or lower *in-vitro* pepsin digestibility than other cereals in raw flour and substantially lower digestibility in cooked products. The reasons why sorghum proteins are less digestible than that of other cereals have not yet been completely elucidated. However, several factors have been identified that may play a role in determining the digestibility of sorghum including: physical grain structure, protein body structure, protein cross-linking, starch properties, and phenolic content/composition of the grain. The majority of proteins in sorghum endosperm are found in digestion resistant spherical protein bodies that have highly cross-linked outer layers. Disulfide bond mediated cross-linking increases during cooking of sorghum, resulting in the formation of highly cross-linked web-like structures of protein. Protein digestibility has a substantial impact on the nutritional properties of sorghum utilization in the production of human foods, animal feeds, and for bio-industrial uses such as ethanol production. The unique properties of sorghum proteins may also influence the digestion of sorghum starch and could play a role in development of low glycemic index foods.

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Introduction

Sorghum (*Sorghum bicolor* L. *Moench*) is thought to have originated in central Africa and was domesticated sometime between 4500 – 1000 B. C (*1*). Sorghum is a member the subfamily Panicoideae in the family Poaceae and is closely related to maize and the various millets.

In Western countries, sorghum's primary use has typically been as an animal feed. Recently, sorghums use in the production of bio-ethanol has seen substantial increases in the U.S. (*2*). However, worldwide, sorghum is used as a basic human food staple for millions and it has been reported that ~ 35% of the worldwide sorghum production is used as human food (*3*). Sorghum is an extremely important food source in areas where food supplies can be limited and hunger is a widespread problem (*4*). Sorghum has been used to produce a wide range of traditional food products as well as 'non-traditional' Western style food products (*5–8*).

Proteins play a key role in the nutritional quality of sorghum for both human foods and animal feed (*9–12*). Sorghum proteins have also been shown to play a role in the production of ethanol from sorghum (*13*) in bio-industrial uses such as production of films (*8*), encapsulating agents (*14*), and in foods (*15*). The goal of this chapter is to provide a review of the structure of sorghum proteins specifically with regard to how the proteins are cross-linked together and how this may influence digestibility and functionality of the proteins in food systems.

Sorghum Protein Overview

Overall Protein Composition of Sorghum

The total protein content of sorghum grain is on par with that present in wheat or maize (*16*). However, variation in cultivation practices such as fertilization, as well as environmental conditions impact protein levels in sorghum. A mean value of 10 percent total protein is typical, with values commonly ranging from 7 to 15 percent (*17*, *18*).

The major protein classes in sorghum grain consist of albumins, globulins, kafirins, and glutelins (*16*, *19*). Nomenclature for determining sorghum protein class has been historically tied to solubility in various extraction solvent systems. Both the Osborne (*20*) and the Landry and Moureaux (*21*) extraction schemes consist of sequential extractions with water (albumin), saline solution (globulin), aqueous alcohol (prolamin), and alkaline or acidic solutions (glutelin). Addition of reducing agents and detergents like sodium dodecyl sulfate (SDS) have allowed further refinement in the isolation of protein fractions exhibiting more specific properties within given protein classes (*22*, *23*).

The albumin and globulin proteins are water and salt soluble fractions of sorghum protein, and are found in greatest abundance in the germ (*24*). Albumin and globulins of cereals have been found to contain proteins that are involved in metabolic processes of the maturing plant and plant defense compounds (*25*, *26*). There is considerable variation in reported relative amount of this protein class, with average levels ranging from approximately 10% to 30+% (*17*, *25*, *27*). Albumin and globulin synthesis in the grain was initiated at 7 days after anthesis

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(*28*) and characterization of these proteins by SDS-PAGE has revealed numerous proteins in the 14–67 kDa range (*24*). The albumin and globulin fractions contain significantly higher levels of lysine than do the prolamins and high lysine sorghum lines have been found to contain higher levels of albumin and globulin proteins relative to normal sorghum lines (*24*, *29*).

Kafirin, a prolamin and the major storage protein of sorghum, has been shown to comprise from 48% to slightly over 70% of the total protein in whole grain sorghum flour (*30*, *31*). Typically obtained by extraction with alcohol and alcohol plus reducing agent, the kafirins are localized in the kernel endosperm in the form of protein bodies (*30*, *32–35*). Of note, over 30% of the total amino acid residues making up kafirin consist of proline and glutamine (*36*). Synthesis of prolamin in developing grain increased from 14 to 28 days after which there was a decline until maturity (*28*). The kafirins function as the major storage proteins of sorghum, and are localized almost exclusively in protein body structures within the kernel endosperm (*35*).

The glutelin protein of sorghum functions as a structural element within the matrix of the peripheral and inner endosperm of the sorghum kernel, as well as a possible source of enzymes involved in starch and protein reserve hydrolysis (*17*, *27*). Beckwith (*37*), and Nucere and Sumrell (*38*) reported glutelin contents made up 40% to 50% of the total protein content of three sorghum varieties. Other researchers (*27*, *39*) found lower levels that ranged from 25% to 34% glutelin. According to Taylor et al. (*27*) such differences may reflect incomplete extractions during preceding sequential extraction steps. Increases in glutelin amounts within the developing grain stopped beyond 14 days after anthesis to maturity (*28*). SDS-PAGE of sorghum glutelins has shown proteins in the M_r of 20–67 kDa (*40*).

Kafirin Subclasses

Interest in the composition of the kafirin proteins of sorghum undoubtedly stems from its prominence as the most prevalent protein component of sorghum endosperm, as well as how it contributes to the nutritional and functional properties of sorghum grain. To lend clarity and consistency to kafirin nomenclature, Shull et al. (*41*) proposed a system for naming kafirin protein subclass components based on the existing maize prolamin (zein) nomenclature system. Factors considered by Shull et al. (*41*) were similarities between kafirin and zein prolamin subclasses regarding molecular weight, solubility, and structure that were confirmed by comparisons using SDS-PAGE and immunological cross reactivity. Three kafirin subclass proteins investigated in that study were accordingly, named α-, β-, and γ-kafirin to correspond with their respective zein counterparts. A fourth kafirin subclass protein exhibiting similarities to δ-zein was identified from cloned sequences of DNA, and is known as δ-kafirin (*36*).

As the major storage protein of sorghum grain, α-kafirin makes up about 80% of the total prolamin protein (*42*). Depending on the research referenced, the molecular masses of α-kafirins are most frequently reported in the 23−27 kDa range (*11*, *36*, *41*). SDS-PAGE analysis resolves the α-kafirins into two bands of M_r about 23,000 and 25,000 (*41*). As an indicator of cross-linking potential, α-kafirins have been reported to contain around 1 mol% cysteine (*11*, *33*).

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Watterson et al. (*42*) reported that β-kafirin composes an average of 7−8% of total prolamin protein. Solubility in 10−60% tert-butanol plus reducing agent as well as cross-reactivity with β-zein provided data for establishing three major M_r components consisting of 16, 18, and 20 kDa (*33*, *41*). In contrast, molecular cloning experiments by Chamba et al. (*43*), found evidence from eight independently amplified clones for a single gene encoding for a 172 amino acid residue β-kafirin species. Their result indicated the presence of 5.8 mol% cysteine as well as an even number (ten) of cysteine residues, and provides evidence β-kafirin may be involved in intra-chain as well as inter-chain disulfide bonding. The high cysteine content and even number of cysteine residues supports evidence indicating β-kafirin is present in predominantly higher molecular weight polymers, and may act as an oligomer chain extender linking other subclass kafirins together (*44*).

The γ-kafirins comprise 9−12% of prolamin protein (*42*) and are soluble using water + reducing agent or 10−80% tert-butanol plus reducing agent (*36*, *41*). Analysis by SDS-PAGE, resulted in a M_r band at 28 kDa coinciding with the migration pattern of γ-zein (*41*). Cysteine content of γ-kafirin has been reported as 7 mol% (*36*) and is an indication of γ-kafirin participation in cross-linking reactions. The participation of γ-kafirin in kafirin cross-linking was demonstrated in studies by El Nour et al. (*44*). Nunes et al. (*45*) showed by comparisons of 60% tert-butanol extracts of cooked and uncooked reduced and non-reduced sorghum that cross-linked oligomers present in greatest abundance were made up of γ- plus α-kafirins.

The δ-kafirins, the final kafirin subclass considered here, have not been extensively characterized at the protein level. Izquierdo and Godwin (*46*) performed molecular cloning experiments of cDNA encoding for δ-kafirin. Predictions from translation of the DNA sequence predicted a 16 kDa polypeptide containing 147 amino acids that contained appreciable (17%) methionine. In addition, Belton et al. (*36*), described two δ-kafirin DNA sequences (GENPEPT AAK72689 and AAW32936) that showed extensive homology with M_r 14 kDa δ-zein.

Organization into Protein Bodies

The organization of sorghum proteins into protein bodies provides insight into the role of grain structure in sorghum nutrition and functionality (*47*). Shewry and Halford (*35*) describe the formation of protein bodies in sorghum and other panicoid cereals as an accumulation of prolamin proteins directly in the lumen of the rough endoplasmic reticulum (RER) leading to formation of individual protein bodies surrounded by a membrane of RER origin. Using transmission electron microscopy (TEM), Taylor et al. (*32*) postulated prolamin polypeptides were synthesized on ribosomes outside the ER before passing directly into the lumen of the RER, thereby forming within the RER membrane. The same study concluded that during grain development, protein body synthesis occurred mainly between the milk stage (14 days) and late hard dough stage (35 days) of grain maturity by measuring the rate of increase in the protein fraction (prolamin) found exclusively

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within protein bodies. Similarly, in another study Johari et al. (*48*) found the most protein accumulation within protein bodies occurring from day 24 to 31.

Physical measurements of protein body structure were differentiated between peripheral and central endosperm by Shull et al. (*33*) using electron microscopy methods. Protein bodies in the peripheral endosperm were 0.3−1.5μm diameter spheroids with intact membranes and a surface coating of ribosomes. Central endosperm protein bodies were irregularly shaped by contrast, and appeared smaller overall yet exhibited a similar absolute range in diameter. Protein bodies at 14 days development were approximately 0.3−0.4μm in diameter, and increased in size by 21 days to diameters of up to 2 μm (*32*).

Early work by Seckinger and Wolf (*49*) indicated sorghum protein bodies were composed of prolamin protein. This was later corroborated in studies involving alcohol plus reducing agent extraction of isolated sorghum protein bodies previously extracted to remove albumin plus globulin (*30*). SDS-PAGE analysis allowed comparison of the prolamin extract from the protein body with equivalent extracts from whole grain of the same sample. The protein bands were identical, thereby confirming that all the sorghum prolamins were located within the protein bodies. The same group determined the matrix surrounding the protein bodies was composed of glutelin protein. Using immunocytochemical localization techniques, Shull et al. (*33*) were able to determine the distribution of the kafirin subclasses within the protein bodies of the sorghum endosperm. Immunoelectron microscopy of protein body regions stained with antibody serum specific for kafirin subclasses indicated peripheral endosperm protein bodies consisted of mostly α-kafirin with minor amounts of γ- and β-kafirins. Central endosperm protein bodies also displayed α-kafirin as the major constituent, but γ- and β-kafirins were present in higher proportion than seen in the peripheral protein bodies.

Differences in Endosperm Types

Sorghum endosperm is characterized by the presence of a hard (corneous, vitreous) endosperm peripheral to a soft (floury, opaque) endosperm located in the kernel center. Scanning electron microscopy studies by Hoseney et al. (*50*) describe hard endosperm as a tightly packed structure without air spaces. The starch granules were tightly packed and polygonal, and covered with a protein matrix covered with protein bodies. In contrast, the soft endosperm starch granules were loosely packed and round, and were interspersed with air spaces. A protein matrix was present, but it was thin and more sparsely covered with protein bodies. The proportions of hard and soft sorghum endosperm vary widely based on cultivar, and may range from all floury to all hard (*17*, *34*).

Shull et al. (*33*) identified the kafirin composition of peripheral (hard) and central (soft) sorghum endosperm by immunoelectron microscopy. The α-kafirins predominated in the peripheral and central endosperm, and although the peripheral endosperm contained small amounts of β- and γ-kafirin, the central endosperm exhibited higher proportions of these two kafirin sub-classes. Selective protein fractionation, SDS-PAGE, and enzyme linked immunosorbent assays were used to determine differences in the protein composition of hard and soft sorghum

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endosperm (*42*). In the same variety of sorghum, hard endosperm contained 1.5-2 times more protein than soft endosperm. In addition, they found the soft endosperm contained less kafirin protein, more albumin and globulin proteins, and that the glutelin fraction was similar in both.

Sorghum endosperm texture is important in many respects including resistance to insect and molds, storage, handling and processing characteristics, and milling properties (*51*, *52*). Relating grain hardness in sorghum and maize to biochemical composition was the subject of a review by Chandrashekar and Mazhar (*34*). The numerous studies reviewed led the authors to conclude that α- and γ-prolamins are usually present in higher amounts in hard grains, as well as in the outer portions of hard grains, as compared to soft grains. Other protein factors involved in grain hardness included increased levels of matrix protein, the presence of more protein bodies, and protein bodies richer in γ-prolamins that are involved in disulfide cross-linking. Ioerger et al. (*53*) sought to differentiate vitreous and floury sorghum endosperm by considering differences in the protein polymer composition of the isolated vitreous and floury fractions. Differential solubility of unreduced fractions was used to obtain molecular weight distributions that were investigated by size exclusion chromatography, free zone capillary electrophoresis, reverse-phase high performance chromatography, and sulfhydryl analysis. In agreement with earlier studies, vitreous endosperm was found to have higher levels of cross-linking and a greater molecular weight distribution than found in floury endosperm.

Protein Digestibility

Protein digestibility in grain sorghum is an important issue with regards to human nutrition, animal feed and even bio-fuels production (*9*, *13*, *54*). Considering sorghum's status as a basic food staple in Asia and Africa, protein digestibility is of major interest with regard to human nutrition. There has been substantial research conducted to determine what factors influence protein digestibility in sorghum (*11*). Various grain components have been reported as potentially hindering protein digestibility including: polyphenols, grain structure, and heating (*11*). A corner stone in the study of protein digestibility in sorghum has been the *in vitro* pepsin digestibility method developed by Mertz et al. (*55*). Virtually all the protein based research below has relied on this method to determine the digestibility of sorghum proteins.

Effect of Heating on Sorghum Proteins and Protein Digestibility

The most intensely studied factor in relation to sorghum protein digestibility has been the effect of wet heating as protein digestibility in sorghum decreases upon cooking. During the cooking process, disulfide links are believed to form either within or between protein chains. These disulfide crosslinks result in the formation of web-like protein structures resistant to digestion enzymes (*15*, *56*, *57*). This is not the case in raw flour. A comparison of raw and wet cooked flour (gruel) showed that protein digestibility of sorghum decreased by ~15%

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when cooked, while maize had a modest increase in 2 of the 3 digestion methods tested (*58*). Maize is closely related to sorghum and is often used to compare to sorghum. Uncooked maize and sorghum had overall similar digestibility demonstrating there were changes in sorghum that occurred during the cooking process which impeded protein digestion. Cooking was found to decrease the extractability of the sorghum prolamins (kafirins) relative to raw flour with the amount of non-extractable protein more than doubled in the digested residue. This study highlighted the role of the alcohol soluble sorghum proteins, kafirins, in contributing to the lower protein digestibility of sorghum relative to maize.

As discussed previously, kafirins have been divided into four subclasses: α, β, γ, and δ. The β- and γ-kafirins contain high levels of cysteine, a source for potential disulfide bonding and are also presumed to be concentrated at the outside layer of the sorghum protein body surrounding the α-kafirins (*36*). This structure has a direct effect on protein digestibility. Oria et al. (*59*) studied the effects of pepsin digestion on the subclasses in cooked and uncooked flour (*59*). A test of extractability of cooked and uncooked flour agreed with the findings by Hamaker et al. (*58*) that cooked flour was less digestible. Oria and coworkers expanded the research on changes in protein solubility during cooking and reported that the β- and γ-kafirins were impacted more by cooking than the α-kafirins. An addition of α-amylase to digest the starch in the flour, along with sonication, was used to promote solubility of the kafirins in cooked samples. In general, starch digestion improved extractability in the β− and γ−kafirins while sonication caused a decrease. However, in this study sonication was carried out for 4 min with no mention of cooling the samples. Thus heat generation during the sonication could have promoted disulfide bonding or protein aggregation. Cooked samples that were reduced and sonicated had slightly higher extractabilities than cooking alone. Gamma kafirins seem to be the most affected by the treatments while α-kafirins varied little among the treatments. Overall protein digestibility of uncooked flour decreased from 69.2% to 43.6% with cooking and after addition of reducing agent digestibility changed from 93.0% to 56.2% respectively. TEM of uncooked and cooked flour with or without reducing agent was also used by Oria et al. (*59*) to visualize the digestion of sorghum protein bodies. The degradation of sorghum protein bodies visually appeared to be at its highest in the uncooked, reduced digested sample. Even the flour cooked in the presence of a reducing agent, sodium bisulfite, and digested showed significant loss of the protein bodies. Oria et al. (*59*) explained that the disulfide bonding of the β- and γ-kafirins on the exterior of the protein body prevented access to the possibly more digestible α-kafirins on the interior. Duodu et al. (*60*) showed that cooking sorghum resulted in the formation of disulfide bonded polymeric protein structures and that more of such aggregates were present in cooked sorghum compared to cooked maize. Nunes et al. (*61*) reported that cooking sorghum and maize led to the formation of high molecular weight aggregates and of particular importance was the formation of two proteins of 45 and 47 kDa that were "nonreducible and nondigestible."

Reducing agents have also been added during the cooking process to cleave disulfide bonds formed while being cooked. Hamaker et al. (*62*) conducted a study using various reducing agents on several types of cereals. It was shown that sorghum flour cooked with 2-mercaptoethanol had the largest increase in

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protein digestibility relative to an untreated cooked sample. Other cereals such as maize, barley, rice and wheat had little change in digestibility when cooked in the presence of a reducing agent. This demonstrated the fact that disulfide bond formation during cooking of sorghum is more relevant to changes in protein digestibility than in other cereals. This is consistent with the results of confocal laser scanning micrographs of cooked sorghum, maize and rice that show only cooked sorghum proteins form web-like structures (*15*). Fermentation has also been shown to increase protein digestibility of raw and cooked flour due to the decrease in insoluble proteins (*63*, *64*).

Relationship between Protein Body Structure and Protein Digestibility

Structural changes in the protein bodies have also been linked to protein digestibility. Recently, sorghum germplasm with increased levels of protein digestibility and lysine have been discovered (*65–67*). These "high digestible" (HD) mutants have similar cooked (~ 85%) and raw (~ 80%) protein digestibility without the addition of a reducing agent (*68*). Evidence of a protein body with various deep indentations as opposed to a circular structure was observed by TEM. The γ-kafirins were concentrated inside the indentations allowing an enzyme better access to the α-kafirins (*68*).

Protein digestibility has also been studied through the maturation cycle of grain (*69*). It was found that as the grain matures the difference between cooked and uncooked flour digestion increases. At 20 days after half bloom (DAHB), digestibility of uncooked and cooked flour was 88.2% and 88.5% respectively while at maturity (after 40 DAHB); it was 73.2% and 55.2%. Comparing kafirin subclasses and percent cross-linking during maturation, there was an increase in cross-linking between 40 DAHB and maturity. At 40 DAHB, the amount of cross-linking was roughly 10%, 10%, and 40% for α-, β-, and γ-kafirins respectively and 30%, 40%, and 80% at maturity. The drop in protein digestion may be affected by the decrease in moisture during development. It could also be the increase of γ-kafirin formation which seems to be the most active in cross-linking. Since γ-kafirins are considered the main actor in protein cross-linking, Lee and Hamaker (*70*) modified a cysteine group, Cys155, of γ-zein to increase its protein digestibility. Zein is the alcohol soluble protein of maize, analogous to kafirin. Because γ-zein demonstrates resistance to digestibility similar to γ-kafirin, studying the properties of γ-zein may help lead to increases in the digestibility of sorghum.

Emmambux and Taylor (*71*) utilized chromatography and spectroscopy to observe the changes in heat treated proteins. Using size-exclusion chromatography (SEC), the authors compared isolated kafirin and zein from uncooked and cooked (both boiling and pressure cooking). It was found that with cooked kafirin samples there was an increase in the largest M_r cross-linked peaks. Minor changes in zein from cooked maize were observed but not nearly as evident as in kafirins from cooked sorghum. This chromatographic method gives a good view of the intact polymeric proteins formed by heating. In SDS-PAGE, the samples are usually reduced and the size of the proteins in their native state is not visible. This discovery demonstrates that isolated kafirin behaves in the same way as do

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proteins in sorghum flour when cooked suggesting that the issue is with the protein and not other factors in the flour. Additional testing using Fourier transform infrared spectroscopy (FT-IR) looked at the possible conformation changes of the proteins with and without the cooking process (*71*). Kafirin and zein samples of isolated protein and flour were observed between 1750 and 1450 cm^{-1}. Zein had little change in structure with cooking except for a broadening in the amide II region, 1575 – 1475 cm^{-1}, showing a slight increase in β-sheet structures. Untreated kafirin had much less β-sheets than α-helices. When cooked, kafirin had almost equal amounts in the amide I region, 1700–1600 cm^{-1}, and more β-sheet structure in the region of amide II. In the indigestible kafirin residues, the β-sheet was the major structure in both amide regions. Emmambux and Taylor (*71*) concluded that the increased ratio of β-sheets to α-helices decreases protein digestibility. Learning the structure of sorghum from the gene level to the grain itself will improve the understanding of what makes sorghum protein digestibility so unique.

Protein Structure and Functionality

As mentioned in the introduction of this chapter, sorghum is a major food staple for millions of people around the world and as such has been utilized in a wide variety of food products, from porridges to fermented beverages to white pan bread. Compared to other cereal grains commonly used for human food (such as wheat), little has been done to study the relationships sorghum endosperm biomolecules and food functionality and end-use quality. The majority of the research that has been conducted in this area has focused on the use of sorghum in traditional foods from Africa and India (which have been divided into several categories for easier study [5]). Several studies have examined the role of sorghum starch and protein in a number of traditional food products (e.g. (*72–77*)). In addition to these studies, research has also been conducted on other types of sorghum food products including: parboiled sorghum (*78*), sorghum tortillas (*79*), sorghum noodles (*80*), snack foods (*81*, *82*), cookies (*83*, *84*), and flatbreads (*84*).

The role of sorghum proteins in food functionality has not been extensively addressed, especially with regards to the production of foods for Western countries. As the majority of the proteins in sorghum are contained in relatively inert protein bodies, their role in food functionality will be limited unless degraded using enzymes, reducing agents, or processing to release the proteins from the protein bodies. However, interest in producing isolated maize and sorghum proteins for use in baked goods has increased since the pioneering work of Lawton (*85*) that showed that isolated maize proteins do posses some visco-elastic properties.

Functionality of Isolated Proteins

Wheat is a unique cereal grain in that its storage proteins are able to form a visco-elastic network or dough. This ability to form visco-elastic protein networks is what allows wheat to form moldable, handleable dough and high quality bake

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goods. Because of the ability of wheat gluten to form dough and high quality baked goods, it is difficult to find substitutes that will function similarly to wheat gluten and provide a quality food source for those with wheat allergies and gluten intolerances. Currently, only a few non-wheat proteins that have some degree of visco-elastic properties have been identified. Proteins of the carob germ and isolated maize proteins have both been found to have gluten-like properties (*85*, *86*). Kafirins have nearly identical physical and chemical characteristics as zein protein, the predominant storage protein of maize. Due to the similarities between kafirin and zein, it is plausible that similar functional traits can be obtained from kafirin.

Because of the parallels between kafirin and zein and the more extensive research completed on zein, zein functionality will be reviewed and used as a model to discuss kafirin functionality. One of the earlier attempts to produce dough from zein protein was completed by Lawton (*85*). In these experiments zein was mixed with starch in a ratio of 10% zein to 90% maize starch to simulate the two predominant fractions of wheat flour (protein and starch). When mixed with water above zein's T_g, zein-starch composite flours were able to produce wheat-like visco-elastic dough. It was found that the visco-elasticity of zein was dependent on the T_g of zein (i.e. temperature and water content). When mixed in a farinograph, dough mixed at 35 °C as opposed to 25 and 30 °C produce the most desirable or visco-elastic dough. Lawton also determined that like wheat dough, formation of visco-elastic zein starch dough was dependent on the development of a network of protein fibers. It was also concluded that below zein's T_g no protein fibers were formed and therefore a visco-elastic dough was not developed.

To follow up on Lawton's work that defined the conditions necessary to form visco-elastic dough from zein and starch, Mejia et al. (*87*) determined the required secondary structures of zein proteins needed to form visco-elastic dough. Like Lawton (*85*), zein was mixed with maize starch in a ratio of 10% zein to 90% maize starch. The zein-starch composite flour was then mixed with water in a mixograph at 35 °C. It was found that substantial changes occurred to zein during mixing and dough formation. Before mixing, the zein was determined to contain ~65% α-helixes and ~30% β-sheets as established by Fourier transform infrared (FT-IR) spectroscopy. In a hydrated visco-elastic dough form at 35 °C, the ratio of α-helixes to β-sheets shifted to ~30% and ~48% respectfully. This demonstrated that the development of a visco-elastic dough from zein-starch composite flour was dependent on temperature (as determined by Lawton (*85*) and shear from mixing. A temperature above zein's T_g coupled with shear in the form of mixing is the causative factor necessary for the increase of β-sheets and decrease in α-helixes needed to form a true wheat-like dough. However, it was found that the β-sheet structures were not stable when compared to that of wheat gluten. As the zein dough was cooled there was a subsequent loss in β-sheet structures and visco-elasticity. Not only was it determined that β-sheets were necessary for zein-starch visco-elastic dough formation, but the quantity of these structures was directly related to dough quality in terms of resistance to mixing as determined by mixograph.

The first research to compare the functionality of kafirin to zein was completed by Oom et al. (*88*). Kafirin was able to form resins similar to that of zein when

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plasticized with oleic acid at a level 50% of kafirin (w/w). Although the kafirins in this experiment were able to form similar resins to zein, the proteins were unable to form visco-elastic dough as described by Lawton (*85*). Instead the kafirin remained as discrete particles in the starch-water matrix. The researchers attributed this to the fact that that the commercially purchased zein contained only α-zeins as determined by SDS-PAGE. Kafirins however, were found to contain both α-kafirin and γ-kafirin fractions in the laboratory extracted protein isolate. The researchers postulated that the γ-protein fraction, found in both zein and kafirin, which may be more hydrophobic in nature when compared to the α-protein fractions did not allow for appropriate hydration of the isolated proteins and subsequent plasticizing when mixed with water and starch. However, data on the hydrophobicity of the various kafirin subclasses is not clear cut. For example, α-kafirins elute much later than other kafirin sub-classes on RP-HPLC (*89*) and are soluble in more non-polar solvents than γ-kafirins (*41*), suggesting that the α-kafirins are more hydrophobic than the γ-kafirins. Another hypothesis made by the researchers is that the cysteine rich γ-kafirin was cross-linking to form disulfide bonds between cysteine residues. This could explain why the resins formed by kafirin were reported to be stiffer and more resistant to extension. It may also help to explain why the kafirin isolates used in the experiment were unable to form visco-elastic dough. The formation of disulfide bonds between and within the proteins may not allow for the appropriate structural changes to occur that were described by Mejia et al. (*87*).

The first breads produced from visco-elastic zein-starch dough were completed by Schober et al. (*90*). To accomplish this, modifications had to be made from the original dough formulation described by Lawton (*85*) and used by others (*87*, *88*). Flour composition for the zein-starch dough and bread was 20% zein 80% maize starch. Hydroxypropyl methylcellulose (HPMC) was also added as a functional ingredient. Other ingredients included water, yeast as a leavening agent, salt, and sugar. The dough was mixed at 40 °C instead of 35 °C. The modifications were made due to the inability of Lawton's (*85*) zein-starch dough formulation to retain enough gas to produce satisfactory bread under the conditions of these experiments. HPMC is a surface active hydrocolloid that not only assisted with gas retention, it also allowed for zein fiber production within the restraints of the experiment. In previous works where zein fibers were identified as being crucial for visco-elastic dough formation, farinographs and mixographs were used to apply shear to the zein-starch water mixture in the form of mixing to create zein fibers (*85*, *87*). Schober et al. (*90*) mixed the zein-starch mixtures by hand, which would have imparted less energy into the zein-starch mixtures used in that study than in the work of Lawton (*85*) and Mejia et al. (*87*). The effect of energy input into zein-starch dough systems has not yet been studying, but it is possibly that mixing conditions may influence the amount of β-sheets and produce a protein network of fibers as identified by Mejia et al. (*87*). Regardless, Schober et al. (*90*) did demonstrate that HPMC could be used to increase the functionality of zein in terms of protein fiber formation and gas retention in dough which was the primary intention of the research. In a latter study by Schober et al. (*91*) the role of lipids in zein-starch dough formation was addressed. The partial removal of lipid from the surface of zein particles was attributed to stronger dough and higher quality bread by possibly allowing increased protein-protein interaction.

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The previous publications discussed above all utilized commercially available zein. While a bulk of the zein isolation process is well known, the proprietary nature of the product leaves many aspects of processing procedure unknown. While the commercially purchased zein definitely does function in the manner described in the various publications reviewed above, it is only indicative of how this particular commercially produced zein functions. For this reason, comparing laboratory extracted kafirins to the commercial available zein protein in terms of functionality should be conducted with caution as the two materials are not prepared in the same manner. Ideally, kafirins and zein should be extracted in an identical manner in order to draw conclusions on differences in their functionality. The commercially available zein should instead be used as a model in identifying the causative factors of both zein and kafirin functionality, so that a reproducibly functional protein isolate can be made in a laboratory setting. Realizing this, Schober et al. (*92*) identified how different isolation procedures impacted zein and kafirin functionality. Relatively pure α-zein isolated in the lab was found to have some properties similar to commercial zein. However, laboratory isolated kafirins did not have the same functionality. One hypothesis put forward by Schober et al. (*92*) was that a-kafirins were more difficult to isolate in a pure form than the α-zeins possibly due to similarities in hydrophobicity between α- and β−kafirins. In RP-HPLC separations, the α− and β−zeins differ substantially in there elution times, suggesting differences in surface hydrophobicity at minimum. It is possible then, that it is easier to isolate α-zeins from β-zeins by controlling the polarity of the extraction solvent. In contrast, α- and β−kafirins elute close together in RP-HPLC suggesting that in sorghum these two kafirin types are more similar in surface hydrophobicity making it more difficult to extract α-kafirins not contaminated with β-kafirins.

Although the experimentally isolated zein was able to aggregate in water this research did not specify if it was able to form visco-elastic dough. The near absence of cysteine in the isolated zein implies that disulfide cross-linking is not responsible for zein resin and dough formation as it is with wheat gluten, but through other interactions. It was suggested that hydrophobic interactions were responsible for zein protein functionality by Schober et al. (*92*).

In order to make kafirin function as commercially available zein, a full understanding in terms of processing and isolation of why commercially available zeins become visco-elastic is needed. It is clear that not all isolated zein is equal in terms of its functionality. Commercially isolated zein from corn gluten meal has been subjected to various processes including exposure to organic solvents, alkaline pH, and high temperatures (*93*). Any of these steps could include modifications to the proteins that are responsible for the functionality of zein. By understanding the cause and mechanisms behind the functionality of isolated zein, they can be applied and modified to form visco-elastic kafirin-starch dough. This could be important for gluten-free food markets and in areas where sorghum proteins could be isolated from flour milling residues (*94*) or dried distillers grains from the bio-ethanol industry (*95*).

In addition to the work described above targeting visco-elastic dough formation, research has been done on modifying isolated kafirins to alter their functionality by creating protein-polysaccharide and protein-protein complexes

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(*96*). A major limitation of this study was in the protein isolation methods used. Proteins were extracted from sorghum using a 0.03 M Tris-HCl buffer, pH 8, containing a reducing agent. By definition, the proteins isolated with extraction procedure were not prolamins and were therefore not kafirins. The proteins that were extracted and used for conjugating polysaccharides and protein onto were most likely a mixture of albumins, globulins and perhaps γ-kafirins, which are known to be water soluble once reduced (*36*). Although no protein yields were reported by Babiker and Kato (*96*), kafirin accounts for ~68-73% of the total protein found in sorghum grain (*31*), hence only a small fraction of the total proteins in sorghum could have been extracted. However, the isolation and modification of kafirins is an interesting concept and kafirins conjugated to polysaccharides or other proteins could have a pronounced change in functional properties and could lead to improved uses of sorghum proteins.

Conclusions

Compared to maize and wheat proteins, sorghum proteins have had substantially less research conducted on their structure and functionality. However, a great deal of information has been gained on sorghum proteins especially through the detailed research of Dr. John Taylor's and Dr. Bruce Hamaker's lab groups as well as others. The most researched area related to sorghum proteins has been to understand the mechanism behind the reduced protein digestibility in cooked sorghum proteins and why this differs so much between sorghum and maize. The unique properties of sorghum proteins have been exploited to produce protein films and encapsulating agents [8], understanding the chemistry and structures of sorghum proteins will likely lead to improving the nutritional quality of sorghum, but may also lead to new uses for these proteins.

Acknowledgments

Names are necessary to report factually on available data; however, the U.S. Department of Agriculture neither guarantees nor warrants the standard of the product, and use of the name by the U.S. Department of Agriculture implies no approval of the product to the exclusion of others that may also be suitable. USDA is an equal opportunity provider and employer.

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Chapter 8

Grain Sorghum Lipids: Extraction, Characterization, and Health Potential

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Grain sorghum (GS) lipid contains valuable phytochemicals that may have potential critical health benefits. Although the chemical composition of GS lipids varies among studies due to different extraction methods, intrinsic factors, such as variety, and the analytical methods applied, it has been consistently reported that GS lipids contain phytosterols, policosanols, unsaturated fatty acids, aldehydes and steryl/wax esters. A series of hamster studies showed that GS lipids exerted health-benefiting effects on cholesterol metabolism and intestinal microbiota. In-vitro experiment with a colon cancer cell line also showed its possible role in cancer inhibition. Exact mechanisms and bioactive compounds related to these effects have not been elucidated. Studies are in progress to understand the possible health benefitting mechanisms as well as to determine the responsible bioactive compounds.

Introduction

Grain sorghum (GS) is consumed as traditional or stable food by many developing countries positioning GS as the 5th most important cereal in the world succeeded only by rice, wheat, corn, or barley (*1*). A major contributor to GS

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world-wide consumption is its tolerance to severe temperature fluctuations and drought conditions allowing production in a variety of eco-agriculture regions (*2*). Grain sorghum research has thereby focused on the development of cultivars for higher yields or for a specific property (*3–5*), and the analysis of each part of the grain (*6–9*), but also includes wax characterization for enhanced production (*10–12*) and sorghum genomics (*13*). Additionally, development of GS flour as a substitute for wheat is increasing because of the growing number of people suffering from celiac disease and other adverse gluten-related health effects (*14*).

The United States currently dominates GS production and distribution, accounting for 20% and 70-80% of the world's supply and exports, respectively (*15*, *16*). Nonetheless, GS is mainly utilized as cattle feed in the US and more recently as a starch source for ethanol production (*17*). Even the lipid-protein rich co-products of dry-grind ethanol production – distillers wet grains soluble, distillers condensed solubles, distillers dried grains (DDG), and distillers dried grains with soluble (DDGS) are distributed solely as animal feed or are disposed. In an effort to increase GS consumption in western cultures, studies proliferated within the past decade have demonstrated the potential health promoting properties of GS either as a whole grain or a source of ingredients (*10*, *18*). These reports show that GS contains a variety of phytochemicals which have been linked to the prevention of many types of diseases, such as cancer, heart disease, and diabetes (*19–23*). Among these compounds, the phenols have been the primary focus, including tannins, phenolic acids, anthocyanins (*24–27*). Yet, recent studies have shown that GS lipids may impart multiple health benefits due to their unique compositional profile (*23–30*). The objective of this article is to review the lipid based research reported on GS and its co-products with an emphasis on the potential health promoting properties.

Grain Sorghum Lipid Composition

Lipid levels present in GS whole kernel (WK) (3.0-4.9%) are comparable to or higher than many other cereal crops commonly consumed in the US, such as corn (4.74%), wheat (1.71%), barley (1.16%), and rice (0.58%) (*31–33*). Further characterization of the GS-WK crude extract (oil + wax) has shown that neutral classes include hydrocarbons, steryl esters, wax esters, aldehydes, alcohols, triacylglycerols, diacylglycerols, monoacylglycerols, free fatty acids, and free sterols (*23*) (Figure 1). Many of these components are abundant in the wax fraction, which constitutes approximately 2:1 to 3:1 of the GS-WK crude lipid depending on the cultivar and extraction method used (unpublished data, (*17*)). As a potential alternative for carnauba, i.e., the most popular plant wax in industry (*34*), GS-WK wax has been extensively analyzed compared to either the oil or the crude extract. Hwang et al. (*35*) and Wang et al. (*17*) reported that GS-WK wax contained mainly fatty aldehydes (44-55%) and policosanols (37-44%), followed by fatty acids (4-8%), hydrocarbons (0.5-0.1%), wax esters/steryl esters (1.4%), and triacylglycerols (0.3-0.4%). Additional analysis of the aldehydes fraction showed saturated C_{28} chains as the primary form (*36*). In contrast, both octacosanol (C_{28}) and triacontanol (C_{30}) accounted for 80% of the policosanols

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fraction while the remaining 20% consisted of hexacosanol (C_{26}), dotriacontanol (C_{32}), lignoceryl alcohol (C_{24}), and nonacosanol (C_{29}) in descending order of abundance (*11*). Comparable to this study, we determined that 86% of the GS-WK policosanol class was comprised of octacosanol and triacontanol while hexacosanol, dotriacontanol and lignoceryl alcohol were detected in lower quantities (unpublished data).

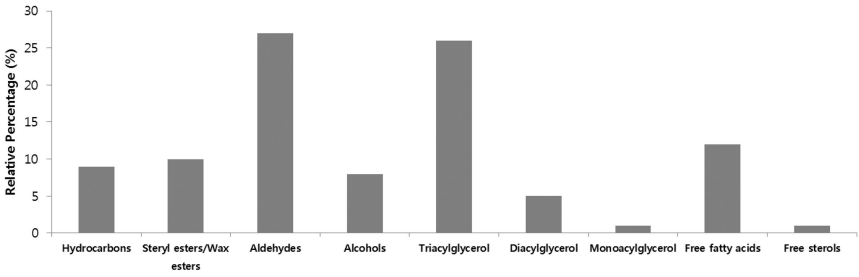

Figure 1. Composition and relative quantities of simple lipid groups in grain sorghum whole kernel (GS-WK). Data from Ref. (23).

Wang et al. (*17*) reported significantly lower wax levels in the lipid fractions of the ethanol co-product, GS-DGG (3.3%), compared to GS-WK (75%) albeit total GS-DGG lipid quantities were higher (9.32%, db). Compositional analysis of the GS-DGG wax showed the presence of wax esters/steryl esters (14.4-17.4%), policosanols (46.3-52.2%), aldehydes (18.4-22.8%), triacylglycerols (0.7-12.6%) and fatty acids (6.3-6.4%) (*11*). Total policosanols levels differed between GS-DGG and GS-WK but each contained octacosanol and triacontanol comprising more than 80% of the policosanol fraction (*11*). Grain sorghum policosanols are novel in that unusually high levels exist as free, nonesterified forms, whereas policosanols in other plants are mostly esterified (*37*).

Similar to other cereal, triacylglycerols is one of the most abundant GS-WK lipid groups (10-25%) (*23*, *31*) and are present in both the wax and the oil (*11*, *17*, *33*). As shown in Table I, linoleic acid, oleic acid and palmitic acid are consistently reported as the predominant GS fatty acids but the relative percentages of each vary, which is most likely due to various extrinsic and intrinsic factors (*6*, *23*, *30*, *38*). Price & Parsons (*39*) showed that the GS fatty acid profile was similar to those of six grains grown in north central U.S. (Figure 2) but Kummerow (*40*) reported that the GS-WK oil was slightly less saturated than corn oil, containing more oleic and stearic acid and less linoleic, myristic, and hexadecenoic acid. Significantly higher levels of triacylglyercols were extracted from GS-DGG (86%) (Figure 3) but the overall fatty acid profile was similar to the GS-WK with linoleic, oleic, and palmitic being the predominant acids (*30*, *38*).

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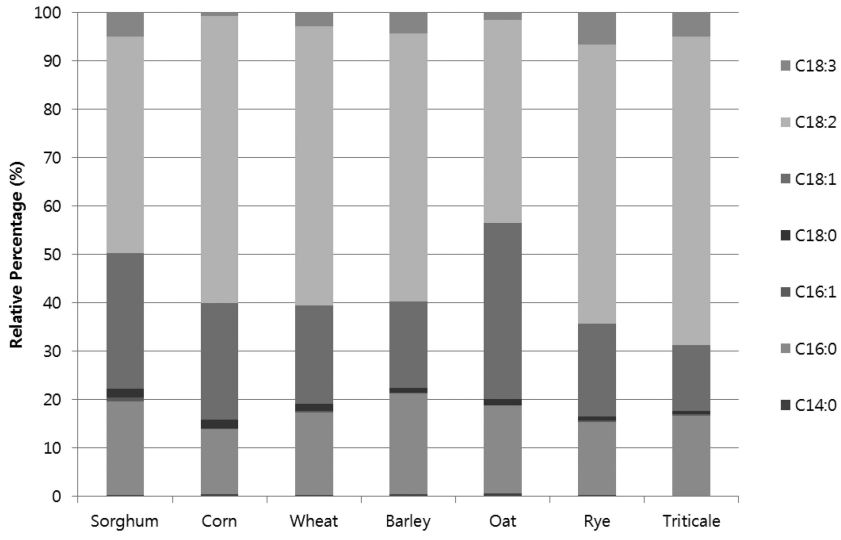

Figure 2. Comparison of fatty acid compositions of seven cereal grains. Fatty acids are named as number of carbons: number of double bonds. Data from Ref. (39).

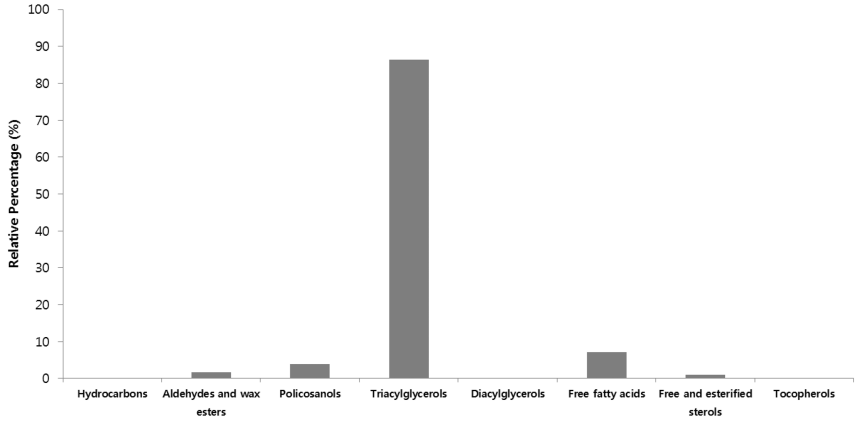

Figure 3. Composition and relative quantities of simple lipid groups in grain sorghum DDG. Data from Ref. (30).

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Table I. Free fatty acid profiles of grain sorghum triacylglycerol

	Carr et al. (23)	*Neucere & Sumrell (6)*	*Wang et al. (38)*	*Osagie (55)*	*Schlegel et al. (Unpublished data)*
Palmitic acid	17%	11.6-13.4%	21.65 ± 2.8 %	13.0-15.3%	22.08%
Palmitoleic acid	Not reported	0.4-1.5%	Not reported	Not reported	Not reported
Stearic acid	2%	2.0-4.0%	Not reported	0.7-1.1%	3.22%
Oleic acid	35%	30.5-41.3%	27.75 ± 1.58 %	21.0-27.3%	31.79%
Linoleic acid	41%	33.2-49.7%	44.64 ± 2.14 %	56.5-59%	37.54%
Linolenic acid	2%	1.8-4.0%	Not reported	2.1-4.0%	3.34%
Sorghum variety	Mixed commercial red grain sorghum hybrids	5 varieties	Mixed commercial grain sorghum hybrids	L187, SSH_3	Macia
Grain form	Whole kernel	Kernel	DDGS	Ground kernel	Whole Kernel
Extract	Crude lipid	Crude lipid	Crude lipid	Crude lipid	Oily fraction
Extraction method	Reflux	Soxhlet	Supercritical CO_2 extraction	Soxhlet	Reflux
Solvent	Hexane	Diethyl Ether	Supercritical CO_2	Hexane/diethyl ether (4:1, v/v)	Haxane

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Reports on GS sterols are limited compared to other lipid components but have become the focus of several groups (*23*, *39*, *41*) due to the increasing evidence linking phytosterols to cholesterol lowering and anticancer properties (*42–45*). As in other cereals, sterols in GS exist in the free and esterified forms (*3*), but total sterol levels are significantly lower than those of corn. In a comparison study, Singh et al. (*46*) obtained 48.4 mg of sterols per 100 g of ground GS compared to the 88.0 mg of sterols per 100 g ground corn. Leguizamon et al. (*41*) also recovered higher levels of sterols from corn DDGS (11.5 mg per g lipid) compared to GS-DDGS lipids (9.79 mg per g lipid). Phytosterol contents in other grains have been also reported by several research groups. When finely ground grains were extracted with hexane/isopropanol (3:2, v/v), total phytosterols in maize, barley, millet, rye, and buckwheat were 43.6, 50.4, 57.8, 75.9, and 106.5 mg per 100 g of the grains, respectively (*47*). Normen et al. (*48*) also reported 28.1, 37.0, 22.5, and 68.3 mg of total phytosterols per 100 g of wheat flour, corn flour, rice flour, and rye flour. Total phytosterols in rye were in the range of 76.1–100.7 mg per 100 g flour when different cultivars and harvest years being compared (*49*). In these reports, β-sitosterol was the most abundant phytosterols followed by campesterol and stigmasterol. Based on studies conducted in our laboratories (unpublished data), lower sterol levels (3.47 mg per g lipid extract) were detected in the GS-WK crude lipid extract than that in the oil fraction alone (8.4 mg per g of oil fraction), which was expected considering the high wax:oil ratio in the crude lipid (Table II). Comparison of GS-WK, GS ground kernel (GK) and GS-DDGS showed that each was composed of the same sterols, including campesterol, stigmasterol, and sitosterol, but at different relative quantities and substantially higher total levels in the GS-DDGS (Table II) (*23*, *41*).

Table II. Phytosterol content and composition of grain sorghum lipids

Lipid type		*WK Crude extract*[1]	*WK Oil fraction*[2]	*DDGS Crude extract*[3]
Total sterol *(per g of GS lipid)*		*3.47 ± 0.09 mg*	*8.41 ± 1.75 mg*	*9.9 ± 1.3 mg*
Relative percentage	**β-Sitosterol**	41%	30%	41%
	Stigmas-terol	33%	35%	42%
	Campes-terol	25%	32%	17%

[1] Data from Ref (*23*), [2] Unpublished data, [3] Data from Ref (*30*).

Tocopherol levels have been cited in the GS literature but the results have not been consistent. The vitamin is either absent or present but at much lower levels reported for other grains (0.71, 0.42, 0.57 and 0.11 mg per 100 g wheat corn, barely and rice, respectively) (*50*). Christiansen et al. (*9*) detected α-tocopherol in only two of the nine GS parent lines tested (21.9 mg per100 g and 57.7 mg per 100 g lipid) whereas Carr et al. (*23*) showed the presence of δ-tocopherol (4 mg

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per 100 g lipid) in a mixture of commercial red GS. Moreover, both α-tocopherol and γ-tocopherol were detected in the GS-DGGS lipid fraction recovered by supercritical CO_2 extraction but the concentrations varied between 0.3-0.7 mg per g of lipid depending on the extraction parameters used (*51*). Zbasnik et al. (*30*) also identified α-tocopherol and γ-tocopherol in GS-DGGS but at 0.20 and 0.19 mg per g of lipid extract, respectively. These inconsistent results may caused by tocols susceptibility to oxidation as affected by storage condition, extraction methodology, and sample preparation (*51–54*).

Reports on complex GS lipids are limited as the main interest has been on the neutral lipids. However, Osagie (*55*) characterized glycolipids and phospholipids isolated from GS-GK by column chromatography and analyzed by thin layer chromatography. The major GS glycolipids were esterified sterol glycoside (38.0%), digalactosyldiacylglycerol (20.9%), and sterol glycoside (10.2%) while lysophosphatidylcholine (41.8%) and phosphatidylcholine (36.6%) were the predominant phospholipids. Lysophosphatidylethanolamine, phosphatidylethanolamine, phosphatidic acid, and phosphtidylglycerol were also present but at much lower levels (*55*).

Analysis Methods for Grain Sorghum Lipid Composition

High quality lipid data generated from any natural system depends on the analytical methods applied, but the unique properties of GS lipids have created additional challenges. In general, GS lipid has been analyzed using commonly used lipid based methods, such as thin layer chromatography (TLC), high performance liquid chromatography (HPLC), and gas chromatography (GC), with more selective methods applied for the identification of components of specific classes or unknown peaks from the first iteration tests. These methods include nuclear magnetic resonance (NMR) and gas chromatography-mass spectrometry (GC-MS).

Analysis of the GS neutral lipid profile has been commonly determined by TLC. For example, Carr et al. (*23*) separated nine lipid classes from the extract of GS-WK samples using a silica TLC plate and hexane, diethyl ether, and acetic acid (85:15:2, v/v) as the resolving solvents. Bands were visualized by submerging the plate in 10% cupuric sulfate and 8% phosphoric acid, followed by charring at 165°C. Zbasnik et al. (*30*) was able to resolve multiple bands by applying the latter method to a crude GS-DDGS lipid extract. In both studies, semi-quantitative data was obtained with densitometry; however, the sample variability was high possibly due to differences in band intensities between plates.

Osagie (*55*) and Price and Parsons (*39*) also used TLC to separate glycolipids and phospholipids in GS. The former researcher resolved glycolipids and phospholipids on pre-coated silica gel plate using two different solvent systems: chloroform, methanol, acetone, diethylamine, and water (120:35:37.5:6:4.5, v/v/v/v/v), and chloroform, acetone, methanol, acetic acid, and water (10:4:2:2:1, v/v/v/v/v), respectively. The bands were visualized by charring with 50% sulfuric acid (*55*). Price and Parsons (*39*) also applied separate solvent systems: chloroform, methanol, and water (75:25:4, v/v/v) for glycolipids and

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chloroform, methanol, water, and 28% aqueous ammonia (65:35:4:0.2, v/v/v/v) for phospholipids. Visualization was conducted on the silica gel plate by spraying with sulfuric acid and potassium dichromate followed by heating (*39*).

Thin layer chromatography of GS wax fractions can be particularly problematic as the wax readily solidifies at room temperature producing precipitates in most solvents resulting in inefficient migration on the plate (*33*). These properties have also caused problems by using HPLC approaches as the wax accumulates on the column and in the lines (*35*). Solubilization of the wax was only achieved after wrapping the exposed HPLC lines with heat strips maintained at 38-40 °C. Major GS wax lipids, including aldehydes and policosanols, were then identified by passing the GS wax fraction through a solid phase silica column followed by injection onto a silica HPLC column interfaced to an evaporative light scattering detector (ELSD). Zbasnik et al. (*30*) used this method for quantitating the major components in the GS-DDGS crude lipid extract. Alternatively, Singh et al. (*39*) were able to detect the major GS lipid classes using either a DiOL column or an alumina column under HPLC normal phase conditions and two modes of detections linked in tandem, i.e., UV followed by ELSD. The DiOL column was able to resolve free phytosterols, ferulated phytosterol esters, free fatty acids, and triacylglycerols while wax esters, sterol esters, and fatty aldehydes were separated by the alumina column.

Gas chromatography has been the method of choice for profiling the fatty acids in various GS lipid matrices (*9*, *23*, *30*). The common approach is to methylate the samples with boron trifluoride in methanol followed by analysis with a 0.25 mm × 30 m DB-Wax capillary column and a flame ionization detector. To further resolve the different chain length policosanols, Hwang et al. (*11*) developed a GC method that consisted of derivatizing the GS lipid extract with N-methyl-N-(trimethylsilyl) trifluoroacetamide prior to analysis with a 0.25 mm × 30 m DB-5 capillary column and flame ionization detector. Alternatively, identification of the different aldehydes' chain lengths was accomplished by passing the GS-WK wax fraction through a silica column and resolving the sample on a preparatory TLC plate by Hwang et al (*36*). The aldehyde band was obtained from the plate and analyzed by GC-MS using a 0.25 mm × 30 m DB-5 capillary column and a MS scan range of m/z = 90-550 amu with further confirmation by ^{1}H NMR and ^{13}C NMR spectra (*36*).

Identification and quantitation of phytosterols from the GS-WK and its co-products has usually been achieved with GC-flame ionization detection method after saponification (*21*, *23*, *30*, *56*). As glycosylated sterol residues cannot be cleaved by this approach, Leguizamon et al. (*41*) obtained higher sterol levels (53.59±4.69 mg per 100 g GS-GK) by hydrolyzing the GS extracts with both acid and alkaline solvents compared to saponification alone (24.85±2.50 mg of sterols per 100 g GS-GK). Using a mixture of bis(trimethylsilyl)acetamide, trimethylchlorosilane and (trimethylsilyl)imidazole (Sylon BTZ) to derivatize the sample, campesterol, stigmasterol, and sitsterol were resolved and detected with a 0.32 mm × 30 m DB-5 capillary column and a flame ionization detector. Christiansen et al. (*9*) detected stigmasterol in GS-GK lipid by using a 0.25 mm × 15 m DB-1 capillary column and a flame ionization detector after derivatization with pyridine and Sylon BTZ, but stigmasterol and triacontanol were not

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completed resolved. As triacontanol is a major GS policosanols, the previously reported stigmasterol content was probably higher than the actual amount. More recently, a GC method capable of separating most neutral lipid classes was applied to GS wax and oil. The method consisted of dissolving a sample in pyridine and derivatizing with N, O-bis-(trimethylsilyl)trifluoracetamide containing 1% trimethylchlorosilane. Analysis with a 0.25 mm × 15 m CP-Sil 8 CB Low bleed/MS capillary column and a flame ionization detector resulted in the resolution of several lipids, including free fatty acids, phytosterols, policosanols, hydrocarbons, and triacylglycerides. Work is in progress to identify peaks in the complex GC profile, especially for the oil fraction (unpublished data).

Extraction Method Effects on Grain Sorghum Lipid Composition

Research has been conducted to recover and purify the main GS lipid components by a sequence of extraction, precipitation, and affinity chromatography on a bench scale (*36*, *57*) but it is still a challenge to separate and purify the potentially high value minor components, such as policosanols, phytosterols, and tocols. From these studies, it has been shown that the GS lipid composition can be affected by different extraction parameters such as method type, solvents, solid to solvent ratio, temperature, time, pressure, and solid particle size. For example, Weller et al. (*12*) compared the yields of the waxy material extracted from the GS-WK using the following hexane-based extraction methods each set at 65 °C for 30 min: 1) reflux, 2) recirculated solvent using the bench-scale laboratory apparatus, and 3) countercurrent extraction with a single pass of solvent through simulated successive stages of extraction. The wax levels were slightly higher when extracted with the countercurrent method (0.200 ± 0.011 g per 100 g dry kernel) as compared to the reflux (0.184 ± 0.003 g per 100 g dry kernel) and the bench-scale recirculated solvent (0.179 ± 0.006 g per 100 g dry kernel) methods. The recirculated solvent method was able to recover comparable levels of policosanol (46.8% of the lipids) relative to the other two methods (45.9% reflux and 43.2% countercurrent) but at a loss of fatty aldehydes (27.4% of the lipids) compared to the reflux method (32.5%) and to the countercurrent extraction (43.6 %) (*12*). Using GS-WK and three hexane based extraction methods (Soxtec, reflux, and bench-scale recirculated), Christiansen et al. (*58*) recovered the highest policosanols levels per lipid extract (~2.5%) with the Soxtec method whereas the refluxing was most efficient in recovering triacylglycerols (~13.5%). Wang et al. (*51*) applied a CO_2 supercritical method to GS-DDGS resulting in higher total lipids (150 g per kg GS-DDGS) and free fatty acids levels (155.3 mg per g lipid) compared to a recirculated solvent method (85 g lipids per kg GS-DDGS and 57.3 mg fatty acids per g lipids) but the sterols and free fatty acids profiles were comparable between both approaches. In another study, Wang et al. (*38*) reported similar lipid yields for a CO_2 supercritical extraction of GS-DDGS (150 g per kg GS-DDGS), which were greater than those obtained using a Soxhlet application (93.2 g per kg GS-DDGS).

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Specific solvents systems of varying polarity have been used to specifically target GS neutral lipids or complex lipids (*3*, *39*, *55*). Hexane-based solvents predominate the GS literature for extracting triacylglycerols, hydrocarbons, sterol esters, fatty acids, monoacylglycerols, diacylglycerols, and sterols (*6*, *11*, *12*, *17*, *23*, *41*, *46*, *58*). Application of same extraction method but alternating diethyl ether as the solvent system, Neucere and Sumrell (*6*) reported different levels of linoleic acid, oleic acid and palmitic acid than those provided by Osagie (*55*) (Table I). In an effort to optimize the extraction of both GS neutral and complex lipids, Osagie (*55*) used different solvent systems with different methods that included Soxhlet and hexane-diethyl ether (4:1, v/v), boiling with 2-propanol followed by methanol-chloroform (2:1 v/v), and boiling with water-saturated butanol. Among these methods, the hot water-saturated butanol approach was most efficient in extracting the polar lipids (Table III).

Table III. The effect of extraction method and solvent system on composition of lipid extracted[1]

Extraction method	*Neutral lipids*	*Glycolipids*	*Phospholipids*	*Total lipids*
Hex/Ether[2]	3.39	0.195	0.04	3.62
CHCl3/MeOH[3]	3.51	0.15	0.63	4.29
Hot WSB[4]	3.38	0.18	0.92	4.48

[1] Each value is the average of the data acquired from two sorghum varieties, which is reported separately in the original article (Ref (*55*)). The unit for all values is grams per 100 g of dry seed. [2] Soxhlet and hexane-diethyl ether (4:1, v/v); [3] Boiling with 2-propanol followed by methanol-chloroform (2:1 v/v); [4] Boiling with water-saturated butanol.

Wang et al. (*17*) compared the yields of GS-DDGS lipid obtained from a bench-scale recirculated hexane method but operating under several conditions, including solvent to solid ratios (2:1 to 5:1), extraction temperatures (45°C, 55 °C, 68 °C), and extraction times (1 to 6 h). Maximum yields were achieved at 68 °C for 4 h (9.32% of the dried GS-DDG) with a solvent to solid ratio of 3:1. In a latter report, Wang et al. (*38*) determined the lipid levels of CO_2 supercritical extracted GS-DDGS in response to varying the mass ratio of CO_2 to GS-DDGS (15, 25, 35, and 45), extraction time (1 to 8 h), extraction pressure (12.5, 20.0, and 27.5 MPa) and temperature (40, 55, and 70 °C). Maximum lipid levels (150 g per kg GS-DDGS) were obtained with a mass ratio of ~45 using a 4 h extraction time at a pressure of 27.5 MPa and a temperature of 70 °C. Lipid yields increased with both pressure and temperature to a point and then the overall lipid began to plateau with increasingly higher pressure and temperature (*38*). However, higher CO_2 flow rates and shorter extraction times on a given mass ratio (CO_2:DDGS) resulted in higher levels of tocols, sterols and policosanols while total lipid yields were not impacted but policosanol yields increased with increasingly higher temperatures.

The particle size of the starting GS is another important parameter to consider when optimizing lipid extraction methods, as reported by Christiansen et al (*58*).

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Using three hexane based extraction methods (Soxtec, reflux, and bench-scale recirculated methods), higher total lipid yields were obtained from GS-GK (1.2-2.9%) compared to GS-WK (0.3-0.6%), which was attributed to the larger surface area of the former sample (*58*) but the GS-WK extracts contained higher levels policosanols. Similar results were reported by Leguizamon et al. (*41*) who showed higher total lipid yields from the GS-GK (2.5-3.0 g per 100 g dry material) compared to GS-WK (0.2-0.3 g per 100 g dry material) using either Soxtec or refluxing and hexane as the solvent system. However, sterols and policosanols extracted from GS-DDGS were respectively 4X and 3X higher than from the GS-GK (*41*).

In summary, a review of the literature showed increased lipid materials, especially those present in the interior of the kernel (triacylglycerols, steryl esters, sterols) with higher solvent to solid ratio, longer extraction times, and smaller particle size (*17*, *41*). Higher lipids yields are also obtained at extraction temperatures near the boiling point of the solvent (*17*).

Intrinsic Factors on Grain Sorghum Lipid Composition

Grain sorghum lipids can be impacted by various intrinsic factors with GS variety being a major effector. Neucere et al. (*6*) showed the lipid yields of five different GS varieties extracted with the same method ranged from 2.66-3.49%. Linoleic acid, oleic acid, and palmitic acid were again the three major fatty acids in all five varieties but the quantities of each contained 33-49%, 30-41% and 11-13%, respectively. The distribution of minor components (behenic, lignoceric, linolenic, and 5-eicosenoic acids) also differed among varieties. Osagie (*55*) compared total lipid levels of two GS cultivars (L187 and SSH_3) developed as long-season and high yielding varieties by using a Soxhlet method with hexane-diethyl ether (4:1, v/v) as the extraction solvent. A final yield of 4.43 g per 100 g was extracted from the L187 cultivar but the SSH_3 cultivar yielded only 2.81g per 100 g of GS. Linoleic acid (56.5-59%), oleic acid (21.0-27.3%), and palmitic acid (13.0-15.3%) comprised more than 95% of the total fatty acids in both varieties.

Preferential distribution of lipid components within the GS kernel has been cited by several research groups. Serna-Saldivar and Rooney (*7*) reported 76.2% of the total GS lipid was located in the germ, 13.2% in the endosperm, and 10.6% in the pericarp with the wax originating mainly in pericarp, which is consistent to the results reported by Karen et al (*8*). In the latter research, wax levels present in the bran (0.3-1.8%), abraded kernels (0.02-0.17%) and the WK surface (0.24%) were also determined. Hwang et al. (*33*) demonstrated that triacylglycerols levels were greater in unpolished GS (223 ± 48 mg per 100 g) due to the higher wax yields compared to polished GS (36.6 ± 5.3 mg per 100 g). However, triacylglycerols accounted for 1.2 ± 0.2 and 5.3 ± 1.7% of the wax fraction extracted from unpolished and polished, respectively. Wax esters/steryl esters (WE/SE) were absent in the GS-WK but were detected in polished GS at 13.3± 3.4% (w/w) (*33*), indicating that WE/SE and triacylglycerols are located mainly in the inner part of the GS kernels rather than on the surface. Lastly, Singh et al. (*46*) separated the germ, fiber, protein, and starch of the GS-WK

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using a wet-milling process, and compared the oils extracted from each part. The germ fraction contained the highest oil levels (13.6%), followed by protein (3.53%), fiber (2.53%), and starch (0.10%) but the phytosterols (0.52%) and their esters (0.32%) were lower in the germ oil than in fiber oil (1.32% and 1.36%, respectively).

Health Effects of Grain Sorghum Lipids

Cardiovascular Disease

Coronary heart disease is a major health concern in the United States, causing 1.7 million hospitalizations and over 600,000 deaths each year, which results in annual costs of $58 billion (*59*). A primary risk factor for coronary heart disease is elevated levels of plasma low density lipoprotein (LDL) cholesterol (*60*, *61*). Yet, relatively little is known about the mechanisms that regulate the input of lipoprotein cholesterol into plasma or the regulatory mechanisms that govern cholesterol absorption from the intestinal lumen into the body. These critical knowledge gaps have hampered the development of treatments that reduce plasma cholesterol concentration. Nonetheless, a link between diet and plasma LDL cholesterol concentration has been firmly established. Lipid based components that have been reported as cholesterol lowering effectors include policosanols (*62–65*), long-chained fatty acids (*66*, *67*), and phytosterols (*42*, *68*). For example, policosanols may play a role in reducing the activity of the rate-limiting enzyme, 3-hydroxy-3-methylglutaryl coenzyme A, in cholesterol synthesis and in increasing the LDL receptor activity (*65*, *69–71*), albeit their overall heart health benefits are under dispute (*67*, *72–74*). As an essential fatty acid, linoleic acid has been linked to LDL cholesterol lowering properties, as shown by the early work of Keys et al. (*75*) and Hegsted et al. (*76*). Other studies have indicated that oleic acid is equally effective in reducing LDL cholesterol as linoleic acid (*77*, *78*). Among various lipid components, the cholesterol lowering benefits of phytosterols have been firmly established. Phytosterols and their esters have been shown to lower both total and LDL cholesterol in blood with the proposed mechanism being the impairment of cholesterol absorption in the small intestine (*42*, *68*, *79*).

As mentioned above, these components have been detected in GS but limited studies have been completed to directly link GS to heart health benefits, especially in human trials. Using a hamster model, however, Carr et al. (*23*) and Hoi et al. (*28*) specifically investigated the cholesterol lowering properties of GS lipids. The initial pilot study was completed with a modified AIN-93M diet supplemented with 0, 0.5, 1.0 or 5.0% GS-WK lipid by weight of diet (*23*). Food intake did not differ significantly for any of the treatment groups during the 4 week feeding periods indicating GS-WK lipid levels as high as 5% were palatable and did not adversely affect animal growth. However, hamsters fed the GS-WK lipid diets presented with significantly lower plasma non-HDL cholesterol in a dose dependent manner (Table IV). Converting the results to percent change relative to the control, the non-HDL levels decreased 18, 36, and 69% in the hamsters fed the 0.5, 1, and 5% GS-WK lipid diets, respectively.

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In addition, plasma HDL cholesterol increased significantly (19%, $p<0.05$) but the only for the treatment group fed a 5% GS-WK lipid diet (Table IV). Liver esterified cholesterol concentration was also significantly reduced in hamsters at each level of GSL intake (Table IV), while GS-WK lipid reduced cholesterol absorption efficiency in a dose-dependent relationship. Carr et al. (*80*) reported that cholesterol absorption efficiency in hamsters was directly correlated with plasma cholesterol concentration and liver esterified cholesterol concentration. Consistent with those results, the study by Carr et al. (*23*) showed significant positive correlation between cholesterol absorption and plasma non-HDL levels ($r = 0.97$, $p = 0.034$) and liver esterified cholesterol concentration ($r = 0.97$, $p = 0.035$).

For the second study, hamsters were again fed an AIN-93M diet but supplemented with the crude GS-DDGS lipids extract (0, 0.5, 1.0 or 5.0%). After 4 wk of feeding, only the 5% treatment group presented significantly lower liver esterified cholesterol levels with no concomitant increases in plasma HDL cholesterol or decreases in plasma non-DHL cholesterol for any group (Table IV) (*28*). Fecal neutral sterol excretion was significantly higher in all treatment groups in a dose dependent manner while no treatment group differed in bile acid excretion (Table IV). The fecal cholesterol excretion was negatively correlated with liver cholesterol concentration ($r = -0.97$, $p = 0.026$) and liver cholesterol concentration was directly correlated with plasma total cholesterol concentration ($r = 0.96$, $p = 0.041$). This evidence indicates that some components in GS lipid modulate plasma and liver cholesterol concentrations by reducing cholesterol absorption and concomitantly increasing cholesterol excretion (*28*). Comparison of the two studies further suggests that the GS-WK lipid was more effective in terms of its overall heart health benefits. Interestingly, the GS-WK extract contained lower levels of phytosterols compared to the GS-DGGS samples (Table II) suggesting that other GS components also act in tandem in lowering cholesterol.

Cancer

Inverse associations between GS consumption and cancer have been reported in epidemiology studies. Chen et al. (*81*) related mortality rates to food-consumption data originating in Shanxi Province, China, which showed esophageal cancer risks were was significantly lower in individuals consuming a GS rich diet. Rensburg (*82*) demonstrated a lower incidence rate of esophageal cancer in African and Asian populations when the dietary staples contained sorghum, while corn and wheat diets were linked to a higher risk for the cancer. Isaacson (*83*) further reported lower squamous carcinoma of the esophagus (approximately 90-95% of all esophageal cancer worldwide) in the South Africans when sorghum was part of the diet and proposed that the changes to corn based diet was the cause for increasing incidence of this type of cancer.

Although studies directly relating GS-lipid consumption to reduced cancer risks are limited, several lipid components present in GS, specifically phytosterols and unsaturated fatty acids, have been studied in isolation. Two studies conducted in Uruguay, one of which was a case-control study and another epidemiology

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study, showed an inverse effect of phytosterols consumption and stomach cancer (*43*) and lung cancer (*84*), respectively. Awad et al. (*44*) investigated the effect of phytosterol on the metastatic ability of human breast cancer cells. This group showed that β-sitosterol not only inhibited the growth of a breast cancer cell line (MDA-MB-231) but also decreased invasiveness of the cells by reducing adhesion of the cells to the components of extracellular matrix. Campesterol did not show the as positive of a response as β-sitosterol. Sasaki et al. (*85*) studied the effect of linoleic acid, the most abundant fatty acid in GS lipid, on gastrointestinal cancer. In their study, linoleic acid suppressed the growth of a human gastric cancer cell line (MKN28) and a human colon cancer cell line (Colo320) in a dose dependent manner and induced apoptosis of those cells. Oleic acid, the second abundant GS fatty acids, was also reported to have an antitumor effect against breast cancer (*84*). When oleic acid was supplemented, Her-2/*neu*-coded p185$^{\text{Her-2/}neu}$ oncoprotein was down regulated in two human breast cancer cell lines (BT-474 and SK-Br3); Her-2 is one of the most important oncogenes in breast cancer (*84*).

Zbasnik et al. (*30*) was the first to directly link anti-cancer properties of GS lipids by using human colon carcinoma cell line (Caco-2) treated with different concentrations of GS-DGGS lipid extract. The GS-DDG treated Caco-2 cells showed decreased cell viability (at 400-1000 g/mL cell suspension) along with increased secretion of the intercellular protein lactate dehydrogenase. Although the latter results suggested a necrotic effect, flow cytometry results showed the GS-lipid could be exerting their effect, at least in part, by inducing programmed cell death (Table V) (*30*). For this study, the GS-DGGS extract was thoroughly characterized containing high quantities of triacylglycerides, sterols, and policosanols among other components in much lower quantities. Additional in vitro and in vivo studies are needed to confirm the anti-cancer effects of GS lipid and to identify the responsible component or multiple components.

Gastrointestinal Health

The mammalian gastrointestinal tract contains more microorganisms than any other surface on the body with the human large intestine alone containing approximately 10^{14} microorganisms (*86–88*). The effects on the micorbiome on host metabolism and diseases have been more intensely studied with the advent of gnotobiotic technology, and especially by comparing germ-free and conventional animals (*89–91*). Association of gut microbiota with diseases has been reported not only in experimental animals but also in human subjects (*92–94*) and studies are on-going to elucidate the underlying mechanisms (*95–98*). Modern molecular approaches have been extensively applied to determine population dynamics and the composition of intestinal microbiota and how it relates to disease (*99–101*). Although remarkably constant within an individual over time (*102*, *103*), the microbiome can be perturbed by diseases and antibiotic treatment (*104–107*) or modulated by dietary interventions such as probiotics and/or prebiotics and fecal bacteriotherapy (*108–110*).

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Table IV. The effect of GS lipid consumption on the cholesterol related metabolism of hamsters

		1st Hamster study (n = 7-8)[1]				*2nd Hamster Study (n = 8-10)*[2]			
		Control	***0.5% GS-WK****	***1% GS-WK***	***5% GS-WK***	***Control***	***0.5% GS-DDGS*** **	***1% GS-DDGS***	***5% GS-DDGS***
Plasma (mmol/L)	**HDL**+	1.93±0.13	1.98±0.13	2.00±0.08	2.29±0.09	2.347±0.0^{b}	2.367±0.1^{b}	2.160±0.1^{b}	1.708±0.2^{a}
	non-HDL	0.87±0.13^{c}	0.71±0.14^{bc}	0.56±0.08^{ab}	0.27±0.05^{a}	1.544±0.1^{ab}	1.746±0.1^{b}	1.569±0.2^{ab}	1.076±0.1^{a}
Liver (µmol/g)	**Free cholesterol**	3.81±0.24	3.57±0.25	3.41±0.22	3.43±0.11	5.88±0.08^{ab}	6.01±0.16^{b}	5.89±0.11^{ab}	5.53±0.12^{a}
	Esterified cholesterol	12.95±1.12^{c}	8.52±1.08^{b}	4.54±0.72^{a}	4.35±0.58^{a}	24.04±1.24^{b}	23.65±1.18^{b}	18.56±3.42^{ab}	12.95±1.81^{a}
	TGÙ	3.12±0.41^{a}	2.88±0.29^{a}	3.16±0.50^{a}	5.37±1.07^{b}	7.06±0.52^{a}	10.05±1.35^{ab}	13.78±1.19^{b}	14.18±2.73^{b}
Feces (µmol × day^{-1} × 100 g^{-1} body weight)	**Bile acids excretion**	0.61±0.10	0.87±0.13	0.86±0.13	0.81±0.18	0.160±0.01	0.129±0.01	0.167±0.03	0.223±0.007
	Neutral sterols	2.68±0.21^{a}	2.87±0.57^{a}	2.93±0.22^{a}	3.95±0.24^{b}	2.495±0.1^{a}	2.996±0.12^{b}	3.476±0.18^{c}	4.140±0.18^{d}

[1] Data from Ref (*23*), [2] Data from Ref (*28*). Values with different superscripts in the same column are statistically differet at $p < 0.05$. * Grain Sorghum whole kernel lipid extract. ** Grain Sorghum dry distillers grain with solubles lipid extract. Ù Triacylglyercides. + High density lipoprotein.

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Table V. Decrease in viability of a colon cancer cell line, Caco-2, with response to GS-DDGS lipid treatment. Data from Ref (*30*)

Concentration of GS-DDGSÙ (µg/ml)	*Percent viability of control[1]*
100	0.66 ± 0.19[a]
200	1.08 ± 0.12[b] *
300	0.84 ± 0.13[bc] *
400	0.71 ± 0.16[ac]
500	0.55 ± 0.10[a]
600	0.47 ± 0.11[a]
700	0.51 ± 0.07[a]
800	0.46 ± 0.08[a]
900	0.47 ± 0.06[a]
1000	0.57 ± 0.06[a]

[1] Percent cell viability as compared to the control (0 µg/ml GS-DDG) measured by MTT assay. Significant differences ($p<0.05$) are designated with different superscripts. * Represents results that are not significantly different from the control. Ù Grain sorghum dry distillers grain with soluble.

The first study to determine lipid dietary effects on the gut microbiome was conducted with GS-WK by Martínez et al (*29*). The authors analyzed the fecal microbiota of male F_1B Syrian hamsters that were fed diets supplemented with 0.0, 0.5, 1.0, and 5.0% GS-WK lipids in a previous study (*23*); and the bacterial populations were analyzed by pyrosequencing of 16S rRNA tags, PCR-denaturing gradient gel electrophoresis, and *Bifidobacterium*-specific quantitative real-time PCR (qRT-PCR). Bifidobacteria increased in response to consumption of GS-WK lipid; a highly positive correlation between total bifidobacteria and plasma HDL cholesterol was shown ($R^2 = 0.75$, $p = 0.0011$). This highly significant correlation, and the fact that HDL cholesterol can be increased through the administration of bifidobacteria (*111*), suggest that the ability of GS lipids to elevate HDL cholesterol is caused through an increase of bifidobacteria. On the other hand, total *Coriobacteriaceae* was decreased with GS-WK lipid treatment, showing highest correlation with plasma non-HDL cholesterol ($R^2 = 0.84$, $p = 0.0002$) (*29*). These results supported other research that showed significant links to cholesterol metabolism and the mammalian host microbiome (*112*, *113*).

The bifidogenic effect of GS lipids is of interest as bifidobacteria are considered health-promoting bacteria that are targeted by current prebiotic strategies. However, additional studies are necessary to determine by which mechanism bifidobacteria are increased. We have shown that GS-DDGS lipids when administered to hamsters as described by Hoi et al (*28*) do not increase bifidobacteria (unpublished data). In addition, when the GS-WK crude lipid extract was separated into oil and wax fractions to further isolate possible bioactive components, no increase in bifidobacteria occurred for either fraction (unpublished data). In this study, the hamsters were fed an atherogenic diet, and an atherogenic diet supplemented with either oil or wax fractions at 5%. Significant

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overall differences among the four treatment groups were presented in phylum TM7, family Lactobacillaceae, as well as unclassified Lactobacillaceae and unclassified Erysipelotrichaceae members, and genera *Lactobacillus* and TM7 *Incertae Sedis* with p-values of less than 0.05 and in phylum Verrucomicrobia, family Coriobacteriaceae, and genus *Akkermansia* with p-values of less than 0.08 (Figure 4 and 5). Interestingly, although the administration of DDGS lipids and the oil fraction of GS lipids did lower total cholesterol levels in plasma, they failed to raise HDL cholesterol levels, potentially associated with their inability to increase levels of bifidobacteria (unpublished data). More studies are in progress to determine the heart health benefits of the GS-oil and wax fraction and its link to modulating the gut microbiome.

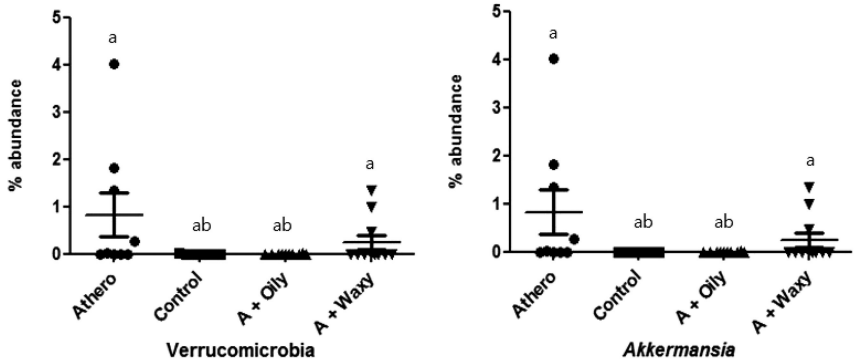

Figure 4. The relative abundance of phylum Verrucomicrobia and genus Akkermansia. Athero, an atherogenic diet; Control, a control diet; A + Oily, an atherogenic diet supplemented with 5% oil fraction; A + Waxy, an atherogenic diet supplemented with 5% wax fraction. Different superscripts are significantly different ($p < 0.09$) using one-way analysis of variance and Tukey's Post-hoc test.

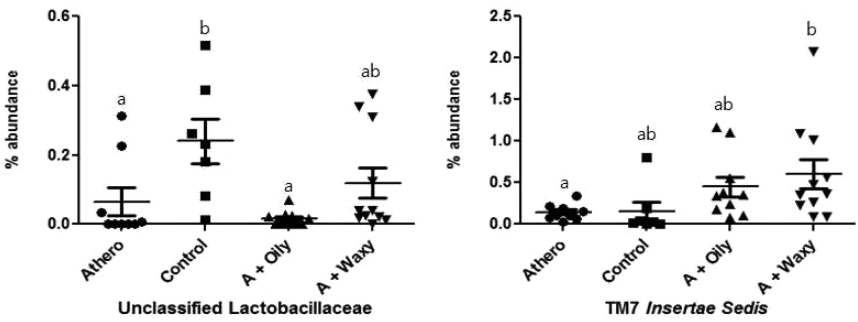

Figure 5. The relative abundance of family unclassified Lactobacillaceae and genus TM7 Insertae Sedis. Athero, an atherogenic diet; Control, a control diet; A + Oily, an atherogenic diet supplemented with 5% oil fraction; A + Waxy, an atherogenic diet supplemented with 5% wax fraction. Different superscripts are significantly different ($p < 0.08$) using one-way analysis of variance and Tukey's Post-hoc test.

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Conclusion

In order to identify and gain further knowledge on the health benefiting effects of GS lipids, a thorough understanding of their chemical composition as effected by cultivar, growth conditions and processing effects is necessary. Therefore, the development of reliable analytical techniques is essential to characterize the unique compositional profiles of GS-lipids. Feedback loops that include agronomics, nutrition, food science and food engineering thus need to be established to continue to enhance GS-lipids as a source of potential health promoting components.

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Chapter 9

Sorghum Flavonoids: Unusual Compounds with Promising Implications for Health

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Sorghum contains high levels of a diverse array of flavonoids, many of which are not typically found in other cereal grains. The high levels of compounds like the stable 3-deoxyanthocyanin pigments, proanthocyanidins, flavones, and flavanones in certain sorghum varieties is especially of interest both from a commercial and health perspective. Evidence suggests that the sorghum flavonoids produce specific health benefits that are not observed for other grains like corn, rice, or wheat. For example, epidemiological and laboratory evidence suggest superior chemoprotective properties of sorghum when compared to other grains. Other evidence indicates that sorghum components elicit anti-inflammatory response and other benefits not seen with other grains in *vitro* and *in vivo*. This chapter reviews the chemistry of the major flavonoids in sorghum, their unusual properties, and potential health benefits.

Part I: Occurrence and Chemistry

Introduction

Flavonoids are widely distributed secondary plant metabolites that have been extensively investigated for their health benefits. They are a class of phenolic compounds that share a basic C6−C3−C6 structure, consisting of two aromatic rings joined by a three carbon heterocyclic ring (Figure 1). More than 5000 flavonoids occur in nature differing in substitution patterns on the benzene rings as well as the heterocyclic ring. In general, flavonoids are grouped based on the heterocyclic ring structure; common classes include anthocyanins, flavanols,

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flavones, isoflavones, flavanones, flavonols, and the polymeric proanthocyanidins. Flavonoids play an integral role in plant growth, reproduction and function. For example, most flower pigmentation in plants (believed to help attract pollinators) is derived from anthocyanins, whereas the high concentration of flavonoids in skin of most fruits, nuts and grains, help protect against pathogens, UV damage, and pests. Flavonoids are also produced inducibly by plants during stress, e.g., pathogen attack, herbivore-induced damage, etc, ostensibly as a natural defense mechanism (*1*).

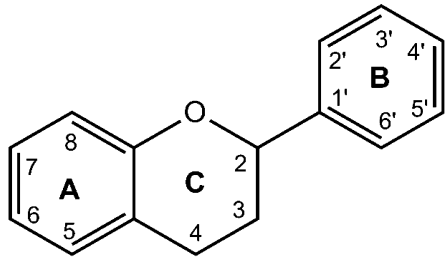

Figure 1. Flavan structure, the backbone of flavonoid compounds; the B-ring attaches at position 3 for isoflavonoids and position 4 for neoflavonoids.

In human nutrition, flavonoids have been long recognized for the important role they play in contributing to health benefits associated with fruit and vegetable consumption. Among the earliest documented health benefits of flavonoids was capillary wall strengthening effect reported by Ruszynak & Szent-Gyorgyi (*2*). The observations led to the term 'vitamin P' being originally used to describe these compounds, probably due to a belief they played the same critical roles as vitamins in human. Besides fruits and vegetables, these compounds are found in relatively large quantities in many food products regularly consumed, e.g., red wine, fruit juices, tea, chocolate, among others. Though research on potential health benefits of flavonoids has been on-going for decades, widespread studies began in the 1990s, when direct evidence for their powerful antioxidant properties (as possible free radical scavengers in the body) became obvious. Consequently, part of health benefits that had been largely attributed to the antioxidant vitamins A and E in fruits and vegetables, or even alcohol (in the case of wine) could be more directly attributed to the phenolic compounds (*3*).

Even with the frenzy of research that began in the 1990s aimed at uncovering the health benefits of flavonoids and other phenolics related to their antioxidant properties in fruits, vegetables and related commodities, few studies focused on investigating antioxidant and health promoting properties of cereal flavonoids. This is partly because cereal grains in general contain low levels of phenolic compounds, especially flavonoids, and most the compounds are discarded with the bran fractions during milling operations. Besides, the health benefits of whole grain consumption were long believed to be largely due to the dietary fiber in bran. Most investigations of cereal grain flavonoids prior to the late 1990s were concerned with their relationship to plant and seed development and resistance

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to various stresses, as well as impact on food product color and other sensory properties. Evidence that phenolic compounds may contribute significantly to the health benefits of whole grain consumption emerged when purified cereal bran fiber components, like cellulose, failed to produce similar effects seen for whole grain.

In sorghum, like most cereals, the phenolic compounds are mostly concentrated in the bran. However, sorghum generally contains much higher levels of flavonoid compounds than most other cereals or even fruits and vegetables (*4*). The type and level of flavonoid compounds in sorghum vary significalty by variety, and are controlled by a set of well documented genes (*5*). Particularly interesting is the fact that the *yellow seed1* (*ys*1) gene found in most sorghum varieties controls the biosynthetic pathway that leads to accumulation of 3-deoxyflavanoid compounds (*6*). These compounds are not usually found in other cereal grains in meaningful quantities, and are partly responsible for the high antioxidant and other beneficial properties of sorghum relative to other grains (*7*, *8*). The unusual flavonoid composition of sorghum and their high levels of accumulation in sorghum bran certainly make sorghum an interesting grain for healthy dietary applications or as a source of bioactive compounds. The chemistry of these compounds and some of their interesting properties are reviewed in the following sections.

3-Deoxyanthocyanins

Of all the sorghum flavonoids, perhaps the most uniquely interesting/ intriguing are the 3-deoxyanthocyanins (Figure 2a). These compounds are responsible for most of the red to black pigmentation on sorghum grain, glumes, sheath, stem, and leaves. Their stability and other attributes have resulted in a growing interest by the food industry to use them as natural food colors. The 3-deoxyanthocyanins are analogous to the anthocyanins (Figure 2b) responsible for the blue, purple and red pigmentation on most fruits, flowers and vegetables. However, unlike the anthocyanins, the 3-deoxyanthocyanins are not substitution at the C-3 position. The 3-deoxyanthocyanins have a very limited distribution in nature, and are typically not present in most other cereal grains or food plants in meaningful quantities.

The synthesis of the 3-deoxyanthocyanin pigments in sorghum is controlled by a set of two genes, *R* and *Y*. A homozygous recessive *yy* will produce a white pericarp essentially devoid of any pigments, whereas a recessive *rr* gene will produce a yellow pericarp with very little 3-deoxyanthocyanins. Dominance at *R_* and *Y_* will result in a red pericarp (*5*); these sorghums accumulate significant levels of the pigments. Other interesting traits have also been observed in sorghum pigmentation; for example, some red sorghum varieties will turn black during grain maturation due to exposure to UV light, with a concomitant many-fold increase in 3-deoxyanthocyanin accumulation (*9*). The genetic basis for this response is not fully understood, but it suggests these sorghum varieties may be producing these compounds as a means of protecting the seed against UV radiation. Sorghum happens to be one of the few species of monocotyledons capable of synthesizing pigmented phytoalexins (secondary

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metabolites produced as a result of mold invasion or other stresses); which are usually the 3-deoxyanthocyanins (*10*). Important differences between the anthocyanins and the 3-deoxyanthocyanins are worth discussing in some detail.

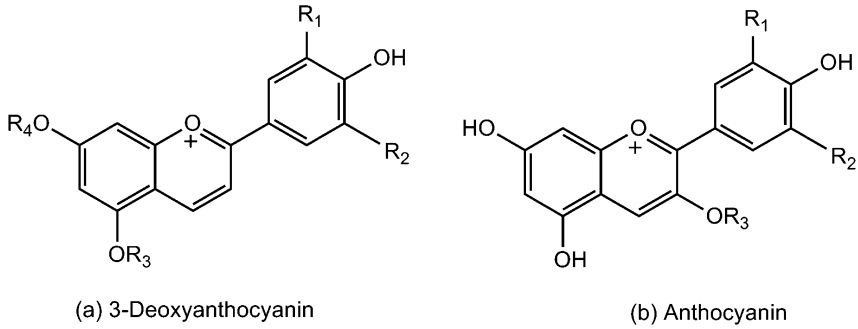

Figure 2. Common 3-deoxyanthocyanin compounds found in sorghum (a) and their analog anthocyanins (b). 3-Deoxyanthoxyanins (a): Apigeninidin based compounds, $R_1 = R_2 = H$; luteolinidin based compounds, $R_1 = OH$, $R_2 = H$; 7-O-methyl derivatives, $R_3 = H$/Glucose, $R_4 = CH_3$; 5-O-glucosides, $R_3 =$ Glucose, $R_4 = H$; Tricetinidin, $R_1 = R_2 = OH$. Anthocyanins (b): Aglycones, R3 = H; glycosides, R_3 = sugar or acyl sugar (C-5 is also sometimes sugar substituted); Pelargonidin, $R_1 = R_2 = H$; cyanidin, $R_1 = OH$, $R_2 = H$; delphinidin, $R_1 = R_2 = OH$.

Chemical Structure and Occurrence in Nature

As previously noted, the most fundamental difference in structure of the 3-deoxyanthocyanins relative to the anthocyanins is the unsubstituted C-3 position (Figure 2a & b). Most anthocyanins in nature are glycosyl substituted (usually with one or more sugar molecules, with or without phenolic acids esters, among other possibilities) at the C-3 position. The glycosyl substitution at C-3 significantly contributes to improved stability of the anthocyanins since their aglycones (with –OH at C-3 position) are highly unstable. By contrast, 3-deoxyanthocyanins tend to maturally exist mostly as their aglycons (3-deoxyanthocyanidins); with lower levels of O-glycysides and acyl-glycoside complexes at position 5 or 7. This may partly be due to the fact that these compounds are rendered relatively stable by the lack of a –OH group at the highly reactive C-3 position, and thus do not need to be glycosylated for stability. The chemical basis for the stability of the 3-deoxyanthocyanins is discussed in the following sub-section.

In sorghum, luteolinidin and apigeninidin (Figure 2a) are the primary aglycones found. These molecules are analogous to the cyanidin and pelargonidin anthocyanidin molecules, respectively (Figure 2b). The structural analogy has allowed for accurate documentation of the effect of 3–OR substitution on the chemical and biochemical properties of anthocyanin compounds over the years. Apart from luteolinidin and apigeninidin, the other major 3-deoxyanthocyanidin

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of dietary importance is the tricetinidin (Figure 2a), a red pigment analogous to delphinidin, commonly found in black tea as an oxidative degradation byproduct of epigallocatechin gallate; this compound has not been identified in sorghum. In sorghum, the two 3-deoxyanthocyanin aglycons, apigenindin and luteolinidin, also exist commonly as their 7-O-methyl derivatives (*11*, *12*), even though 5-O-methyl forms have been reported (*13*). These O-methylated compounds generally have very similar chemical properties (e.g., stability and color absorption characteristics) to their base molecules, but somewhat different bioactive properties that may be relevant to sorghum variety selection for health applications (*12*) (see Section II). It is important to note that for widely distributed anthocyanins found in grains and other plants that are O-methylated, the substitution is typically on the B-ring, as is the case for 3′,5′-O-dimethyl substituted malvidin and the 5′-O-methyl substituted petunidin. However, uncommon anthocyanidins, including hirsutidin, and rosinidin (found in *Catharanthus roseus*) with O-methyl substitution on the A-ring have been found in some plants.

A few glycosides and acyl-glycosides have been identified in sorghum, even though many of these compounds are yet to be structurally identified judging by the number of unknown peaks on HPLC chromatograms we have observed for various sorghum pigment extracts. Presence of 5-O-glucosides of apigeninidin and luteolinidin were originally reported by Stafford (*14*). The authors also suggested presence of acyl-glycosides of the 3-deoxyanthocyanidins in sorghum; this was later confirmed by Hipskind et al (*15*) who identified a ferulic acid ester of arabinosyl-5-O-apigeninidin in sorghum. Polymeric 3-deoxyanthocyanins have also been reported in sorghum, though the precise structures are unknown. More recently, Khalil *et al* (*16*) isolated a symmetrical pyrano-3-deoxyanthocyanidin pigment (pyrano-apigeninidin-4-vinylphenol) from a red sorghum (Figure 3). Obviously the growing interest in the sorghum pigments for food and health applications will lead to increased research into unraveling new details about the composition of these compounds.

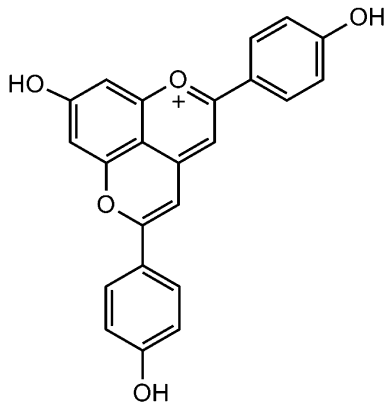

Figure 3. A Pyrano-apigeninidin-4-vinylphenol recently identified in sorghum (16).

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In sorghum, the levels as well as relative proportions of apigeninidin and luteolinidin-based compounds is influenced by variety/genetics. For example, red pericarp sorghum grains with tan secondary plant color have much higher proportion of apigeninidin-based compounds, with only trace amounts of luteolinidin-based compounds whereas sorghums with rep/purple secondary plant color have a more balanced distribution of luteolinidin and apigeninidin-based compounds (*9*, *17*). Specialty pigmented sorghums generally contain higher levels of pigments than other pigmented cereals (Table I), which points to an economic potential of sorghum as a source of natural food colorants.

Table I. (3-Deoxy)Anthocyanin content of pigmented sorghum relative to other cereal grains

Commodity	*Amount (μg/g)*	*Reference*
Black sorghum	1,000 – 2,800	(*18*)
Red sorghum	14-680	(*9*, *17*, *18*)
Lemon yellow sorghum	8-108	(*19*)
Black sorghum bran	4,700 - 16,000	(*18*, *20*)
Blue barley	4	(*21*)
Red/purple maize	225 - 965	(*21*)
Blue/purple wheat grain	35-507	(*21*, *22*)
Blue/purple wheat bran	108-485	(*23*)
Black rice	158-299	(*24*)

Accumulation of 3-deoxyanthocyanin pigments in sorghum grain is almost exclusive to the pericarp, as is the case for other cereal grains; this implies the sorghum bran which is in most cases a byproduct of grain processing could significantly increase in value as a concentrated source of these compounds. However when physiologically stressed, e.g., via fungal attack during grain maturation, the sorghum may synthesize a high concentration of the phytoalexin 3-deoxyanthocyanins at the site of attack, some of which may leach into the endosperm causing a blotchy appearance. This is a type of grain weathering, and is usually more apparent in sorghum cultivars with red or purple secondary plant color. Recent evidence indicates that purple plant sorghum sheath has an order of magnitude higher level of the 3-deoxynathocyanins than found in bran (*25*). In fact these sorghum sheaths are widely used in West Africa as a source of commercial cosmetic dyes. Interest in using them for food applications is currently strong.

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An important difference between the 3-deoxyanthocyanins and anthocyanins is the color properties. The 3-deoxyanthocyanins absorb visible light maximally at lower wavelength (typically below 500 nm at pH below 7.0) than the anthocyanins (usually above 510 nm). Thus the 3-deoxyanthocyanins appear yellow – orange whereas the anthocyanins appear red – blue. The two groups of compounds are consequently not necessarily substitutable, but would likely complement each other in food applications. However, an especially important distinction of the 3-deoxyanthocyanins is their relative resistance to a drop in molar absorptivity (color fading) as the pH increases when compared to anthocyanins. Both anthocyanidins and 3-deoxyanthocyanidins exist in the form of the orange-red flavylium cation (AH^+), red-blue quinoidal base, and the colorless carbinol pseudobase and chalcone species (Figure 4). The relative concentrations of these species and the final color of the solution depend on the pH.

The predominant form of both anthocyanidins and 3-deoxyanthocyanidins at pH $\leq$1.0 is the AH^+; this is also the form that has the highest molar absorptivity (produces most intense color). The AH^+ becomes less stable as pH increases and transforms into the various forms via hydration (into colorless carbinol and chalcones) or deprotonaton (into quinoidal forms). The degree of transformation depends on the solution pH, ionic properties, as well as the structure of the (3-deoxy)anthocyanin molecule. Thus the maximum color intensity and stability of (3-deoxy)anthocyanin compounds is achieved in strongly acidic environments where the AH^+ is most stable.

As the pH increases, the color intensity of (3-deoxy)anthocyanin pigments drops significantly. For example, cyanidin lost 84%, while pelargonidin lost 95% of its absorbance when the pH changes from 1.0 to 3.0 (*17*, *18*) (Table II). In fact the monomeric non-acylated anthocyanins lose almost all their absorbance at pH 4 – 5, due to near complete transformation of the flavylium cation to the colorless chalcones and carbinol pseudobases. On the other hand, the predominant sorghum 3-deoxyanthocyanidins, luteolinidin and apigeninidin were reported to lose only 20% and 38% of molar absorptivity as pH changes from 1.0 – 3.0, and retained significant absorptivity at pH above 4.0 (*17*, *18*). Additionally, the 3-deoxyanthocyanins are relatively resistant to color degradation over time in presence of anthocyanin bleaching agents like ascorbate or sulfites (*26*, *27*). Co-pigmentation, particularly with ferulic acid and tannic acid were found to markledly improve their stability further (*28*).

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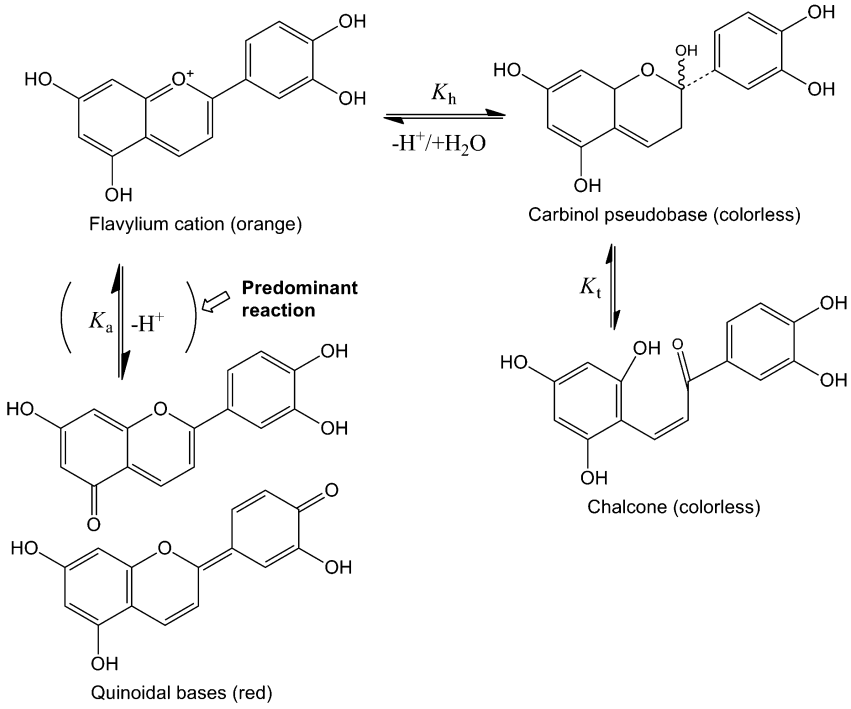

Figure 4. Transformation reactions of luteolinidin in aqueous solution at pH above 2.0. Due to the higher deprotonation constant (K_a) relative to hydration constant (K_h) of 3-deoxyanthocyanidins, the predominant reaction as pH increases is the deprotonation to colored quinoidal bases which help enhance color stability in mildly acidic solutions. Anthocyanins on the other hand, hydrate much more readily losing most color at pH 3 – 5.

The better stability of the 3-deoxyanthocyanins to pH change and hydrophilic attack compared to the anthocyanins has been attributed to the relatively hydrophobic nature of the heterocyclic ring of the 3-deoxyanthocyanins. For one, these compounds have lower hydration rate constants than ionization rate constants (*29*, *30*); this implies that in aqueous solution, they more readily deprotonate as pH increases into the quinoidal bases which are themselves colored compounds. On the other hand, anthocyanins, which have on average three orders of magnitude higher hydratation rate constants than the 3-deoxyanthocyanidins (*29*) are more prone to hydrophilic attack and thus transform mostly into the colorless carbinol bases as pH increases. In addition, the AH^+ of the anthocyanins loses stability at lower pH than the 3-deoxyanthocyanins. For example, in an experiment by Mazza and Brouillard (*29*) cyanidin-3,5-diglucoside hydrated readily at pH above 0.5 and existed as AH^+ and carbinol forms at 50% and 35% (pH 2.0), and 15% and 80% (pH 3.0), respectively, in equilibrium. The quinoidal forms did not reach 10% even at pH 6.0 in this experiment. On the other hand, equilibrium distribution of 3-deoxyanthocyanidins were markedly different under

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similar conditions; for example, apigeninidin existed almost exclusively as AH^+ at pH 2.0, and as 85% AH^+ and 10% quinoidal bases at pH 3.0 (*29*, *30*). Thus it seems the relative hydrophobic nature of the 3-deoxyanthocyanidins results in a poor hydration of the pyrylium (heterocyclic) ring and contributes to their protection in aqueous system at lower pH.

Table II. Effect of increase in pH on relative molar absorptivity of 3-deoxyanthocyanidins and their anthocyanidin analogs. Adapted from Awika et al (*17*)

pH	*3-deoxyanthocyanidins*		*Anthocyanidins*	
	Luteolinidin	*Apigeninidin*	*Cyanidin*	*Pelargonidin*
1.0	100	100	100	100
2.0	97	96	47	42
3.0	80	62	16	05
5.0	36	41	13	00

Values shown are percent absorbance normalized to absorbance in pH 1.0 buffer. Molar absorptivity based on absorbance at λ_{max} for each sample. Values of λ_{max} (nm) in pH 1.0 buffer: Luteolinidin, 482; apigeninidin, 468; cyanidin, 516; pelargonidin, 504.

Opportunities and Challenges of Using Sorghum Pigments as Natural Colorants

Even though sorghum pigments are relatively stable when compared to commercially available ones from fruits and vegetable (e.g., red cabbage) (*27*), there are practical hurdles that need to be addressed for these pigments to become commercially more feasible. For one, the compounds are still susceptible to bleaching at higher pH by ascorbic acid or bisulfites (both relatively common food additives), though to a lesser extent than the anthocyanins. However, new discoveries are being made on the pigment reactions and natural conformations that will significantly enhance their stability in presence of food additives. For example, in presence of pyruvic acid (and possibly acetic acid), the sorghum 3-deoxyanthocyanins form pyrano- structures that are very resistant to, for example, ascorbic acid bleaching. This is because the cyclic condensation reaction between C-4 and the -OH at C-5 blocks the C-4 position that is most susceptible to nucleophilic attack by ascorbate/sulfites. This same mechanism is believed to contribute to stability of wine pigments. The recent discovery of natural pyrano- forms of 3-deoxyanthocyanins in a red sorghum variety (Figure 3) is very interesting in this regard.

Another problem with sorghum grain pigments is their extractability. The pigments in bran are generally very difficult to extract in aqueous solvent under atmospheric conditions. In fact for most research reporting, sorghum pigments are often extracted in acidified methanol, which will extract at least 90% more

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pigments than acidified water under similar conditions (*31*). Even then, most of the pigments remain in the bran, judging by the dark color left on residue even after soaking for 24 hr in acidified methanol. This implies that the reported values for pigment content of sorghum bran are highly underestimated. Improving pigment yield from sorghum bran in water often requires temperature and/or pressure manipulation. With growing commercial interest, newer more efficient extraction methods will undoubtedly be devised, e.g., use of cellulosic enzymes to break down the bran and easily release the pigments may become more feasible by borrowing from the rapid advances in biofuel-based cellulosic digestion technology. On the other hand, pigment extraction from non grain sorghum tissue, such as glumes or sheath, is much easier and can be readily achieved in water at room temperature. Given that glumes and sheath from purple plant sorghum contain especially high levels of the 3-deoxyanthocyanins (*32*, *33*), they are a logical target for easy concentration of these compounds for commercial applications.

A third practical problem is related to the stability of these compounds; as previously explained, the 3-deoxyanthocyanins resist degradation in large part due to their resistance to hydration. This unfortunately leads to a higher tendency of these molecules to self associate and precipitate out of aqueous solution over time, particularly as pH rises to above 4.0 when the quinoidal forms become dominant. This may make the 3-deoxyanthocyanins less appealing for some applications. However, various mechanisms, some that are already widely used in the industry, are available to address this problem.

In all, the sorghum pigments are likely to contribute significantly to the natural food colorant market, especially as companies work to transition from the petroleum based synthetic dyes to the natural food colors, necessitated partly by consumer demands and more importantly by government regulation (especially in the European Union). Within the past 5 years or so, the use of natural colorants has sky rocketed, and in 2010 accounted for about one third of the more than $1.5 billion food colorant market. However, given natural colorants cost 10 – 20 times more than their artificial counterparts, the room for growth is enormous. Sorghum has added benefits in terms of higher concentration of pigments (Table I), better stability to storage, and ability to derive pigments from parts of grain/plant that are typically low value feed/waste. The fact that the sorghum pigments provide hue properties that are complementary to the anthocyanins and other natural colorants increases their importance.

Flavones

Flavones are a group of flavonoids that contain a 2-phenyl-1-benzopyran-4-one skeleton (Figure 5a). Flavones are mainly reported in herbs such as parsley and celery (*34*). However, sorghum accumulates flavones and their derivatives at nutritionally significant levels (Table III). In sorghum the two major flavones present are apigenin and luteolin, along with their 7-O-methylated derivatives (*9*, *14*), and some glycosides. The fact that sorghum flavones are mostly apigenin and luteolin derivatives points to a possible common biosynthetic pathway of these compounds and the 3-deoxyanthocyanins. Accumulation of flavones is sorghum is

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highly influenced by genetics; pigmented sorghum grain with tan secondary plant colors have much higher levels of these compounds than pigmented sorghums with red/purple secondary plant color. For example, tan plant sorghums with a pigmented pericarp had 60 – 386 μg/g flavones, whereas purple plant varieties with a pigmented pericarp had 3.5 – 47.1 μg/g (Table III) (*9*, *19*). The levels of the flavones in the tan plant sorghum varieties is on average an order of magnitude higher than values reported for common sources like celery (*35*). Thus the flavones in sorghum are likely to contribute significantly to health benefits of sorghum, particularly the tan plant varieties.

Apart from sorghum, a diverse array of flavones, including tricin, orientin, vitexin, among others, have also been identified in other cereal grains, though generally at low levels. Among the grains that contain significant levels of flavones are wheat, millet, oats, and fonio. In fact fonio (*Digitaria exilis*) was reported to contain relatively high levels of luteolin and apigenin (150 and 350 μg/g, respectively) (*36*).This is partly attributed to the relatively large proportion of pericarp (where most flavonoids are concentrated in grains) in the grain; fonio is a very small seeded grain with 1000 kernel weight of only 0.5 – 0.6 g, compared to 20 – 35 g typical for sorghum.

Table III. Flavone and flavonone levels (μg/g) in sorghum grains of different plant secondary color and pericarp color

Plant color	*Pericarp color*	*Flavone levels (μg/g)*	*Flavanone levels (μg/g)*	*References*
Tan	White	19.4	nd	(*19*)
Tan	Lemon yellow	268	1,428	(*19*)
Tan	Reddish/brown	60 – 386	8.1 – 48.4	(*9*, *19*)
Purple/Red	Lemon yellow	24.8 – 47.1	694 – 1,780	(*19*)
Purple/Red	Reddish/brown	3.5 – 20.8	26.0 - 238	(*19*)
Purple	Black	36.2 – 40.9	trace	(*9*)

Values based on 12% moisture.

Flavonols

Flavonols share the same backbone structure with flavones, except for the hydroxyl group at C-3 of the flavonols. Thus the flavonols are 3-hydroxyflavones (alternatively, flavones can be viewed as 3-deoxyflavonols, much like the relationship between anthocyanins and 3-deoaxyanthocyanins) (Figure 5b). Various flavonols have been identified in cereal grains. However, these compounds are relatively rare in sorghum and have only been sparsely reported. Original report of flavonol in sorghum was by Nip and Burns (*37*), who identified kaempferol-3-rutinoside-7-glucuronide in a red pericarp sorghum. More recently quercetin 3,4′-dimethyl ether was identified in non-grain sorghum tissue

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(*38*). Again, the limited evidence of flavonols in sorghum suggests sorghum biosynthetic pathways favor the 3-deoxyflavonoids over the 3-hydroxyflavonoids (*6*).

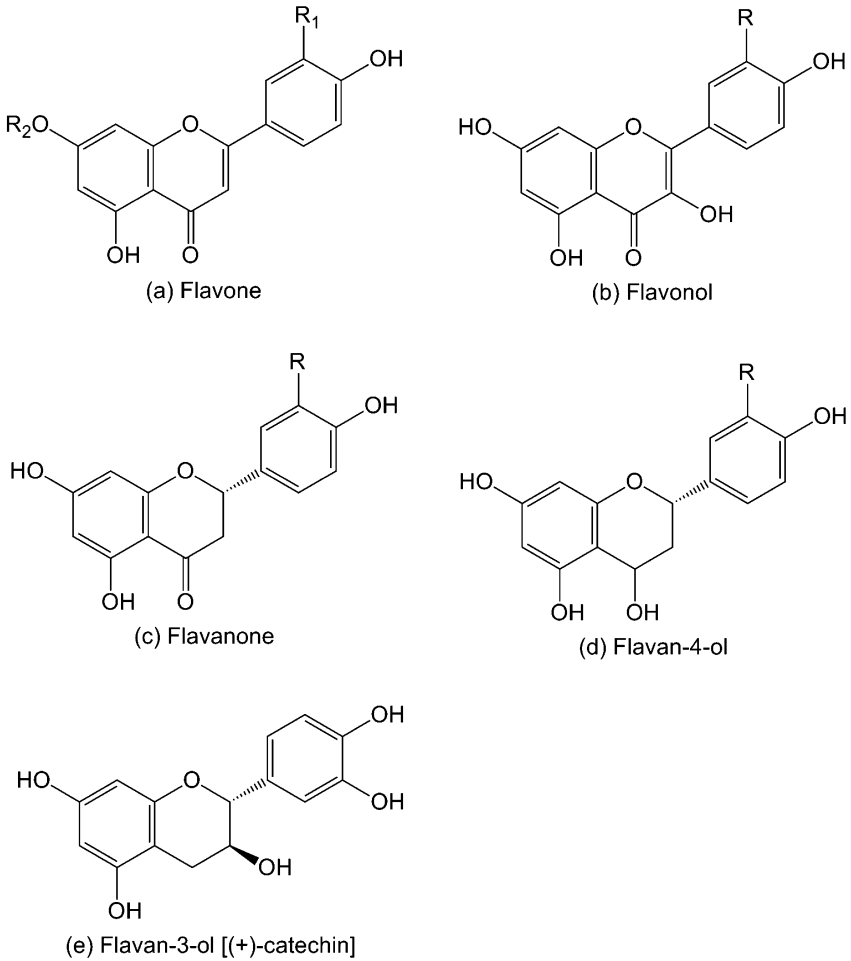

Figure 5. Major classes of monomeric non-3deoxyanthocyanin flavonoids found in sorghum. Flavones (a): Apigenin, R_1 = H, R_2 = H; luteolin R_1 = OH, R_2 = H; 7-O-methyl derivatives, R_2 = CH_3. Flavonols (b): Kaempferol, R = H; Quercetin, R = OH. Flavanones (c): Naringenin, R = H, Eriodictyol, R = OH; 5-O-glycosides and 7-O-methyl derivatives of these compounds are also found in sorghum. Flavan-4-ol (d): Apiforol, R = H; luteoforol, R = OH.

Flavanones

Flavanones have the basic 2,3-dihydroflavone structure, i.e., differ from flavones by the lack of a double bond between C-2 and C-3 (Fig 5c), they thus

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have one chiral center at the C-2 position. Flavanones are widely distributed in nature since they are key intermediates in flavonoid biosynthetic pathway. In food plants, they are however most readily associated with citrus as the major dietary source. For example, naringenin in grapefruit has been widely studied for its health as well as potentially adverse pharmacological effects. Among grains, sorghum accumulates relatively high levels of flavanones (Table III). The major flavanones identified in sorghum are primarily eriodictyol and naringenin as well as their glycosides (*39–41*). We have also detected 7-O-methyl derivative of naringenin, as well as other unknown flavanones in some sorghum varieties.

Like other flavonoids, levels of flavanones in sorghum is highly influenced by genetics, especially genes coding for pericarp color, with apparently minimal influence of secondary plant color (*9*, *19*). Lemon yellow sorghums have the highest levels of flavanones regardless of plant color (Table III). In fact the levels of flavanones reported in these sorghum varieties (694 – 1,780 μg/g) are much higher than those reported for citrus (including grapefruit) fruits which range between 400 – 600 μg/g on a fresh weight basis ((*19*, *42*). Thus the flavanones in the lemon yellow sorghums are likely to contribute significantly to any health benefits derived from consuming these grains. However, as of yet, no studies have reported bioactive properties of the lemon yellow sorghum cultivars.

Even though secondary plant color does not affect overall levels of flavanones in sorghum, it seems to affect the type of flavones present (*9*). These authors reported that the predominant flavanones in the purple plant sorghums was naringenin and its derivatives, accounting for up to 80% of the total flavanones; while eriodictyol and its derivatives were more significant in the tan-plant sorghum varieties. The precise mechanisms by which genetics control the accumulation of the flavanones in sorghum and the specific genes involved need to be investigate further. Since the lemon yellow sorghums typically contain very low 3-deoxyanthocyanin levels, it is possible that silenced downstream genes in the flavonoid pathway lead to the unusual accumulation of the flavanones. Likewise, increased accumulation of 3-deoxyanthocyanins (as seen in black sorghum) leads to negligible levels of flavanones in sorghum (Table III). The potential health implications of these sorghums as well as possibility to use their bran as a concentrated source of the flavanones should be investigated. For example, the flavanones in sorghum bran fractions are 4 – 6 times the levels found in the whole grain (*19*).

Flavan-4-ols

Flavan-4-ols are flavone-derived alcohols (Figure 5d). In sorghums, flavan-4-ol compounds are synthesized from flavanones, naringenin and eriodictyol, and are considered precursors of 3-deoxyanthocyanins (*43*, *44*). They include luteoforol (leocoluteolinidin) and apiforol (leucoapigeninidin) and are believed to play an important role in mold resistance in sorghum (*45*, *46*). These compounds are common monomeric units of condensed tannins in sorghum, and can exist in significant quantities in some varieties. For example, Bate-Smith and Rasper (*47*) reported up to 900 μg/g luteoforol in sorghum grain. In fact initial reports suggested that most sorghum tannins are polymers

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of luteoforol (proluteolinidin) and apiforol (proapigeninidin) as opposed to the typical procyanidins found in other plants (*47*, *48*). This may be true for some varieties; however, later reports suggest a greater diversity in sorghum proanthocyanidins. In general sorghums with tan plant color have lower levels of flava-4-ols than the purple/red plant sorghums.

Flavan-3-ols

Flavan-3-ols (also sometimes simply referred to as flavanols) are a subclass of flavonoids that contain 2-phenyl-3,4-dihydro-2H-chromen-3-ol skeleton (Figure 5e). Flavan-3-ols are widely distributed in fruits, vegetables, and other food plants like tea and cocoa beans, and are the main building blocks for procyanidins (a form of condensed tannins). The most common flavan-3-ol monomers found in food plants include catechin, epicatechin, and epigallocatechin. In cereal grains, barley and sorghum are the major commodities that contain catechin and epicatechin, along with their polymers. In sorghum these compounds are mostly found in their condensed polymeric form (*49*).

Proanthocyanidins (Condensed Tannins)

The group of flavonoid compounds most readily associated with sorghum are traditionally the condensed tannins. Adverse effect of tannins on nutrient digestibility in animal feeding rations is well documented, and has been the subject of countless studies. Breeding efforts to select sorghums with no condensed tannins (to improve feed efficiency) over the years have been largely successful, resulting in very limited production of tannin sorghums at present. Condensed tannins (also referred to as proanthocyanidins because they release anthocyanidins on acid hydrolysis) are polymeric forms of mainly flavan-3-ols and flavan-4-ols and their derivatives (Figure 6). The degree of polymerization ranges from 2 to more than 10. The polymerization typically occurs via C4→C8 interflavan bonds, referred as a B-type linkage (Figure 6a – c). Additional ether bonds between C2→C7 can exist in some of the tannin polymers producing what is referred to as the A-type linkage (Figure 6d). These A-type tannins have been associated with health benefits in cranberries (*50*).

In cereal grains, condensed tannins are rarely accumulated in the seed, and have only been reported in sorghum, barely, and millets. In sorghum, the tannins initially identified were the 3-deoxyproanthocyanidins, proluteolinidin (*14*, *47*) and proapigeninidin (*48*). However, a greater diversity in composition of sorghum tannin polymer has been documented over the years, with catechin, epicatechin, gallocatechin, epigallocatechin, eriodictyol, along with glycosides of some of these monomers as chain extenders or terminators (*41*, *51*, *52*). However, whether sorghum genetics is associated with the accumulation of specific structural conformation of the tannins is not known.

In sorghum, the tannins typically accumulate in a discrete layer known as a pigmented testa, which is sandwiched between the endocarp and the aleurone layer parts of the pericarp. The genetics of tannin accumulation in sorghum is well documented. Generally sorghums without the pigmented testa do not contain

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tannins; the presence of the pigmented testa is controlled by $B_1\_B_2\_$ genes (*5*) . Dominance at both loci is required for a pigmented testa to be present; for example, $B_1\_b_2b_2$ or $b_1b_1B_2\_$ will not have the pigmented testa, and thus will not contain any tannins. In addition, sorghum is classified into three types depending on the levels of tannin present in the grain; type I sorghums do not contain any tannins (lack a pigmented testa). Type II and III sorghums both have the $B_1\_B_2\_$ genes, and thus contain tannins. However, type III sorghums also contain a dominant $S\_$ gene (spreader gene), which makes the pericarp color phenotypically brown (*53*) and levels of extractable tannins (up to 68 mg/g reported) (*54*) much higher than in type II sorghum (typically 0.2 – 8.0 mg/g). Thus tannin-containing sorghums are commonly referred to as 'brown' sorghum.

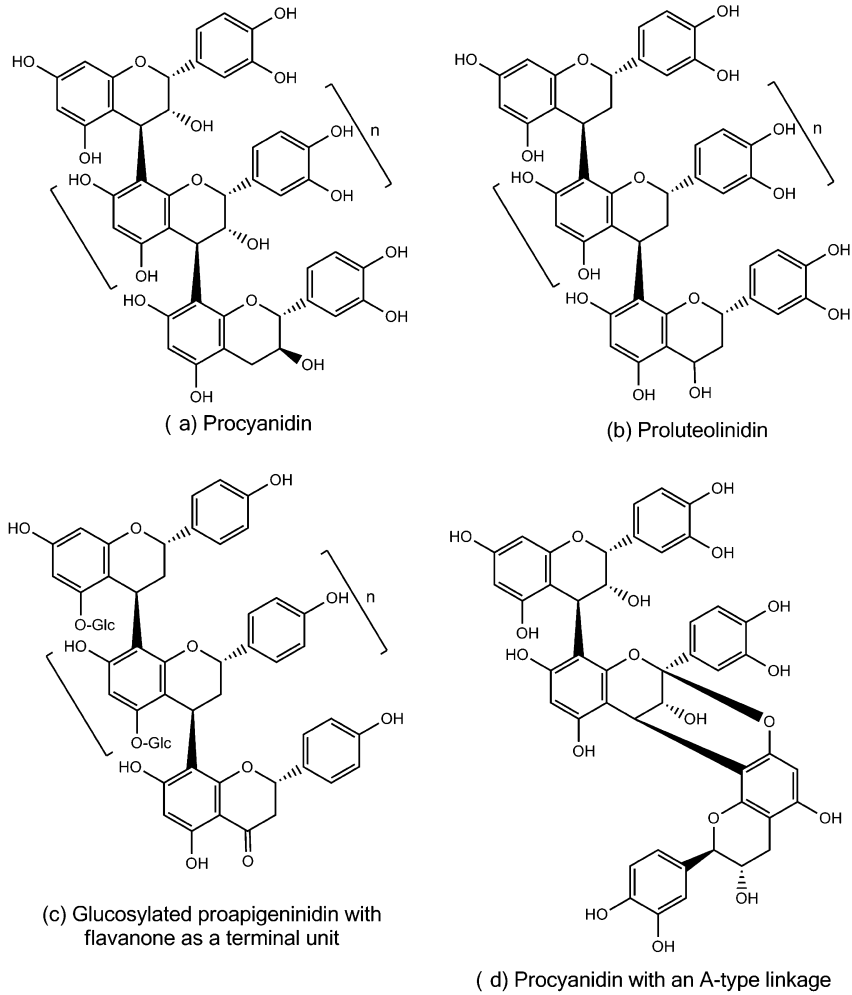

Figure 6. Some proanthocyanidin (condensed tannin) structure identified in sorghum. For dimers, n = 0, otherwise n = 1 – >10. Glc; glucose.

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Tannins in general are structurally very diverse, and derived their name from their ability to tan leather, which basically involves the tannins binding to collagen protein. Tannins are also well known for their interaction with salivary proteins which causes the feeling of astringency commonly experienced when consuming wine, tea, fruit skin, etc. The interaction between tannins and proteins is believed to be an important part of plant defense against herbivores, bird depredation, among others. In fact, tannin sorghums are commonly referred to as "bird resistant" sorghum, since birds will generally not consume them if other food options are available. Sorghum tannin binding with proteins is highly selective and involves both hydrogen bonding and hydrophobic interactions. Proteins that are rich in proline with an open structure and relatively large molecular weights bind preferentially with tannins (*55*, *56*). Proline residues disrupt the protein α-helix structure, leading to an open conformation with carbonyl and amide groups extending outwards, thus maximizing opportunity for hydrogen bonding and hydrophobic interactions.

The tannin-protein binding is usually very strong, and such complexes are largely indigestible. In addition tannins are good metal chelators, and thus can significantly reduce bioavailability of multivalent minerals like iron and zinc. A large body of evidence accumulated over the years has demonstrated significant effect of feeding tannin sorghums on suppressed weight gain by monogastric animals. The effects are attributable to not only nutrient binding, but also inhibition of enzymes as well as intestinal brush-border bound amino acid transporters. However, the tannin interaction with various dietary components is today largely considered a plus, particularly in regards to human health, in terms of reducing dietary caloric intake, increasing antioxidant status in the gastrointestinal tract, among others (*54*, *57*).

Part II: Health Properties

Introduction

Increased whole grain consumption is universally recognized as one of the best ways to improve human health. Whole grain consumption is associated with reduced risk of various chronic diseases, including cancer, heart disease, obesity, diabetes, among others. Even though dietary fiber, as well as important vitamins and minerals in whole grain contribute to some of the benefits, it is clear from numerous studies that the overall benefits cannot be explained by these components alone. It is obvious that the flavonoids and other phenolic antioxidants concentrated in cereal brans are important contributors to health benefits of whole grain. As mentioned previously, whole grains are in general low in phenolic compounds, especially when compared to the so called 'super fruits'. However, grains are much more widely consumed on a consistent basis, and in higher quantities than fruits and other high antioxidant products, and are thus likely to produce tangible benefits from their antioxidants. Additionally, grains are high in bound phenolic compounds that likely contribute more of the antioxidant-related benefits *in vivo* than can be measured *in vitro*.

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The levels of flavonoids and other phenolic compounds in sorghum, on the other hand, are much higher than most grains and rival those in the 'super fruits'. This makes it likely that sorghum will contribute to the health benefits associated with whole grain antioxidants at much reduced levels of consumption. Data on specific health benefits of sorghum flavonoids is still relatively limited, but growing with the expanding consumer interest in whole grains and 'ancient grains'. We summarize some of the data documenting the potential benefits of various flavonoids found in sorghum and their implications in the following sections.

Antioxidant-Related Mechanisms

Given the central role oxidative stress plays in chronic disease development, most investigations of health benefits of various foods commodities invariably begin with antioxidant assays. For a long time, key dietary vitamins, A, C and E have been known to share free radical scavenging as one of the mechanisms by which they protect humans from disease. The recognition that most fruits and vegetables that were associated with health benefits also contain high levels of phenolic compounds that had antioxidant capacity similar to or more powerful than that of the vitamins, led to the overall shift of perspective from the vitamins and minerals as the primary source of health benefits associated with fruit and vegetable consumption. Even though most flavonoids are generally poorly absorbed in the small intestine (estimated at about 5% on average), the overall consumption is relatively high compared to that of the antioxidant vitamins. For example, is estimated that daily consumption of flavonoids in the typical American diet is about 210 mg/day (*58*); with people who consume tea regularly, the levels can be as high as 800 mg/day, according the Pauline Institute of Oregon State University. By comparison, average daily intake of vitamins C is less than 80 mg/day, while that of vitamin E is less than 10 mg/day.

In most cereal grains, reported antioxidant activity is generally low when compared to fruits and vegetables; this is partly due to their low levels of flavonoids and extractable phenolics in general. As stated earlier, most phenolics in cereals are strongly bound to cell wall material and cannot be extracted in typical organic solvents used in antioxidant assays. However, sorghum stands out among cereal grains owing to its high levels of flavonoids (for example, Tables I and III). In fact, levels of flavonoids in sorghum exceed those reported for high dietary phenolic sources like fruits and tea. For example, tea, which contributes the bulk of dietary flavonoids in the American diet, contains 130 – 300 mg flavonoids (mostly in the form of catechins) per 235 mL cup (*59*). By comparison, type III sorghums contain 28 – > 60 mg catechin equivalents (CE)/g proanthocyanidins, with levels in bran exceeding 150 mg CE/g (*20*). With a reported approximate 50% loss during cookie processing (*49*), cookies made with 5 g type III sorghum bran each could supply more than 350 mg CE/cookie. In addition, the sorghum also contains significant levels of other flavonoids, like 3-deoxyanthocyanins, flavones, etc, that would contribute significantly to overall flavonoid intake.

It is thus not surprising that sorghum has been shown to have much higher free radical scavenging activity when compared to other cereals grains, or even

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fruits and vegetables (*54*). For example pigmented sorghum varieties show 10 – > 20 times the antioxidant activity of red wheat (*20*). The antioxidant activity in various sorghum varieties is strongly correlated with their phenol and flavonoid content (*7*). Among all varieties of sorghum, tannin sorghums have the strongest antioxidant capacity; this is attributed to the generally higher free radical scavenging power of tannins relative to simple flavonoids (*20*, *57*). Condensed tannins have more hydroxyl groups in close proximity to each other and are thus more effective at quenching peroxyl radicals than simple phenolics, and are not capable of acting as pro-oxidants via redox cycling (which is sometimes the case for simple phenolics) (*57*). These authors, also proposed that even though tannins are largely non-absorbable due to the large molecular weight and strong complex formation with macromolecules, they likely serve an important role as free radical sinks in the gastrointestinal tracts, thus sparing other dietary antioxidants, in addition to potentially protecting the gastrointestinal epithelial cells. The same could be logically said for the bound phenolic compounds in cereal brans (which form the majority of grain phenolics). They likely contribute more to the antioxidant properties of whole grain in the gastrointestinal tract than assumed.

Apart from direct free radical scavenging, various bioactive compounds, including some phenolics, are capable of stimulating synthesis of various endogenous antioxidant and detoxifying enzymes, e.g., glutathione reductase, quinone oxidoreductase, among others. However, such properties are not directly related to antioxidant activities of phenolic compounds as such, but more to their specific structural conformation. For example, recent evidence demonstrates that O-methyl substituted 3-deoxyanthocyanidins in black sorghum induce the phase II enzymes, quinone oxidoreductase in murine hepatoma cell model *in vitro* (Figure 7), which suggests ability to enhance phase II detoxifying enzymes in humans (*12*, *60*). Such specific activity was not observed for non-methoxylated 3-deoxyanthocyanidins or other flavonoid groups in sorghum, and has not been reported for other cereals. Enhance activity of the phase II enzymes has been linked to chemoprevention, among other benefits. This implies that specialty sorghum varieties that accumulate the specific active molecules could be selected for targeted health applications.

Another common effect of many flavonoid antioxidants is anti-inflammatory activity. Chronic inflammation, which is directly related to oxidative stress, is believed to be a common pathway to various chronic diseases. Thus ability of bioactive compounds to keep inflammation in check is considered one of the most important predictors of health promoting potential. Emerging data suggests that sorghum flavonoids, and maybe yet unidentified compounds in sorghum, are especially potent anti-inflammatory agents. For example, bran extracts from various sorghum varieties, including tannin sorghum, and non-tannin sorghum with red, black and white pericarp inhibited the activity of hyaluronidase; over-expression of hyaluronidase can lead to chronic inflammatory conditions responses in macrophages and dendritic cells (*62*). Brans from tannin and black sorghum also reduced the release of cytokines after LPS (lipopolysaccharide) irritation *in vitro*, and reduced infection induced by TPA (12-O-tetradecanoylphorbol acetate) in rats (*63*). The anti-inflammatory effect correlated with phenolic content and antioxidant capacities of the brans.

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Interestingly, wheat and rice brans did not show anti-inflammatory properties in each of the two studies. This suggests that the unique flavonoids in sorghum may be driving the observations. Another study (*64*) demonstrated that white sorghum bran extract suppressed IgE production by U266 cells, whereas wheat bran extract did not; the authors were unable to identify the compound(s) involved. Additional evidence indicates that sorghum pigments extracted from sorghum stem eliminated the oxidative stress in rat brain induced by cyclophosphamide which may suggest protective effect from oxidative stress-related neurodegenerative diseases (*65*).

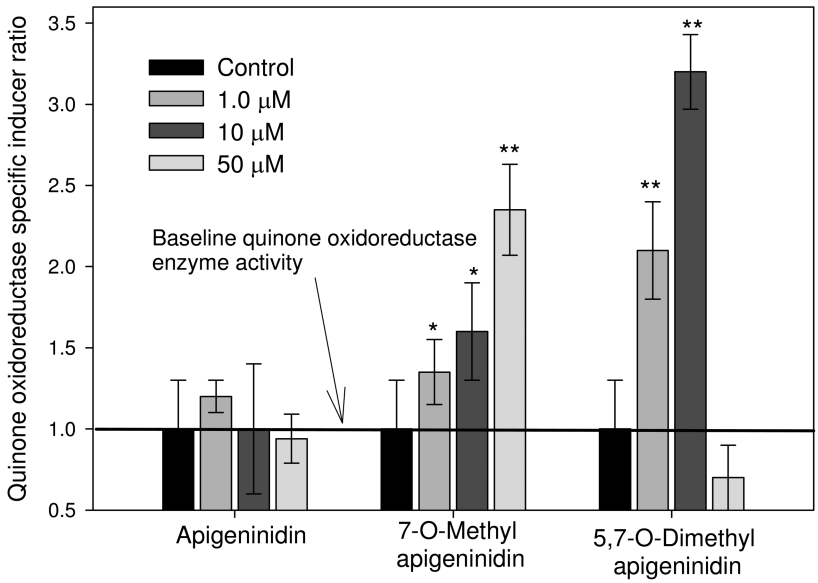

*Figure 7. NAD(P)H:quinone oxidoreductase specific inducer capacity of apigeninidin and its methoxylated derivative found in black sorghum compared to synthetic dimethoxyapigeninidin. Hepa1c1c7 murine hepatoma cell lines were used for the assay. Cells (10,000/well) were induced with extracts for 24 h before assay. Error bars represent ±sd from three separate experiments. *, P < 0.05; **, P < 0.001, compared to control (Bonferroni multiple-comparison test). Adapted from (11, 61).*

Indirect evidence is also available for various flavonoids found in sorghum. Quercitin (*66*), naringenin (*67*), luteolin and apigenin (*68*), all found in sorghum, were shown to reduced the formation of prostanoids and leukotrienes during lipid peroxidation, by inhibiting eicosanoid generating enzymes, such as phospholipase A2.

Obesity, Glycemic Response, and Related Properties

Obesity (and related health problems) is undoubtedly one of the greatest health challenges facing mankind today. Until fairly recently, obesity was considered a problem exclusive to the developed world. However, cases of obesity and diabetes are increasingly inflicting developing world at rates that rival those in the developed world. For example, according to Organization for Economic Co-operation and Development (OECD), Mexico currently rivals the USA for the top spot of obesity incidences in the world. Unfortunately, the poorest of developing countries have not been spared either. It is not uncommon to find overweight and starvation coexisting side by side in many countries in Africa, for example. The fact that most weight loss/weight management strategies peddled over the past two decades by both credible and dubious entities have not produced much impact adds to the magnitude of the problem. It seems human beings have become victims of their own success; food production has improved by leaps and bounds in the past century, while technological advances and wealth accumulation mean that physical exertion is optional for an increasing segment of society.

Sorghum has been long known to have reduced feed efficiency relative to corn, particularly type III tannin sorghums. In particular, monogastric animals seem especially susceptible to type III tannin sorghum; reduced feed efficiency and weight gain by 10 – 50% have been reported when tannin sorghums (containing 25 – 35 mg CE/g) were fed to rabbits, pigs or chicken (*69*–*71*). In all these experiments, the feed efficiency of non-tannin and low tannin (probably type II) sorghums was identical to that of corn. Thus even though sorghum proteins (especially kaffirins) are known to cross-link during processing and slow digestibility of carbohydrates, it seems their influence on feed efficiency is modest at most.

The best documented mechanisms by which sorghum tannins reduce nutrient digestibility include binding of food proteins, and possibly carbohydrates, into non digestible complexes as previously explained. Such complexes become part of dietary fiber and may actually play an important role as antioxidants in the digestive tract (*57*), besides possibly modulating colon microflora. Besides directly binding to macro-nutrients, tannins can also bind to digestive enzymes including, amylases, proteases and lipases (*72*, *73*), thus inhibiting their activity. Additional mechanism reported for sorghum tannins is via inhibition of intestinal brush-border bound amino acid transporters (*74*). Since these effects have not been reported for sorghums with the monomeric flavonoids, it is apparent that the polymeric tannins are the ones involved. There is no direct evidence for tannin sorghum consumption and glycemic properties or nutrient digestibility in humans. However, anecdotal evidence cited by Awika and Rooney (*54*) indicates that some cultures in Africa prefer tannin sorghums due to their long satiety effect, likely related to slowed nutrient digestibility by tannins.

Given the important role sorghum plays as a staple in many developing countries, strategies to increase its productivity and food use in these regions should be encouraged. As the developing world becomes increasingly aware of connection between diet and long term health, the use of traditional foods

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like sorghum that had been abandoned to a large extent in urban middle class for the more 'westernized' diets will increase. However, providing scientific concrete evidence of whether and how sorghum consumption can contribute to weight management and incidences of diabetes will be crucial. In addition, such evidence would stimulate food use of sorghum in the rest of the world, especially as a component of calorie control dietary strategies. However, impact of tannin sorghums on micronutrient bioavailability, particularly zinc and iron, must be considered, especially in populations that are deficient in these nutrients.

Cancer

Positive correlation between whole grain consumption and cancer prevention is well documented; the evidence is especially strongest for gastrointestinal cancers. For example,Larsson et al 2005 (*75*) reported that colon cancer risk was reduced 33% in women who consumed 4.5 servings of whole grain per day compared to those who consumed less than 1.5 servings per day. Whole grain consumption was also associated with a 50% reduced risk for upper digestive tract (esophagus, oral cavity and pharynx) (*76*). Countless other studies support these findings. Whole grain contributes these benefits likely through a combination of factors; including antioxidant activity, particularly of the bran cell wall-bound phenolic compounds; soluble and insoluble dietary fiber which help regulate digestion as well as colon microbiota; and via phytoestrogenic properties contributed by lignans and other estrogenic compounds in whole grain.

Among cereal grains, sorghum has had the most striking evidence for its potential to benefit chemoprevention. Van Rensburg (*77*) reported reduced incidences esophageal cancer in various parts of the world (Africa and Asia) where sorghum consumption was high, whereas consumption of maize and wheat correlated with elevated incidences. The authors proposed that sorghum may act by mitigating the adverse effects of micronutrients deficiency that increased the risk of such cancer in these areas. An epidemiological study by Chen et al. (*78*) revealed similar findings for Sachxi Province, China. This study included 21 communities within the province over a period of 6 years and found that regions that consumed highest amounts of sorghum, and to a lesser extent millet, had 1.4–3.2 times lower mortality from esophageal cancer than areas that primarily consumed wheat or corn. The authors corrected for consumption of other foods like alcohol, tea, meats and vegetables. Growing incidences of squamous carcinoma of the oesophagus among blacks in South Africa was attributed to the change in diet from sorghum to maize (*79*). Available evidence clearly suggests that there is something in sorghum that contributes to cancer prevention over and above what other grains may provide. It has been suggesested that the apparent chemoprotective effects of sorghum is due to the fact that sorghum has much lower incidences of aflatoxin relative to, say corn. However, the fact that other grains like wheat which are also very low in aflatoxin don't show similar benefits suggets that there are actually active components in sorghum that contribute the benefits.

Emerging evidence with cell culture models does demonstrate that there is indeed more to the unique sorghum flavonoids than mere speculation. Shih et al

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(*8*) compared the two primary 3-deoxyanthocyanidins in sorghum, luteolinidin and apigeninidin, with their analog anthocyanidins, cyanidin and pelargonidin, respectively, for ability to inhibit cancer cell growth *in vitro*. They found that luteolinidin and apigeninidn were much more effective at reducing cancer cell proliferation than their analogs at all the concentration tested. For example, at 200 μM concentration, luteolinidin inhibited HL-60 leukemia and HepG2 liver cancer cell proliferation by approximately 93% and 47%, respectively (Table IV). At equivalent concentration, cyanidin inhibited the cells by 24% and 0%, respectively. Yang et al (*12*) reported relatively potent inhibitory effect of apigeninindin and luteolinidin and their various derivatives against HT-29 colon cancer proliferation, with many of the compounds inhibiting cell growth by 50% at below 50 μM. The authors reported that O-methyl substitution improved the inhibitory properties of the 3-deoxyanthocyanin compounds, with 5,7-dimethyl substituted forms being most active. They also reported in the same study that black sorghum pigment extract was much more potent at inhibiting the HT-29 cell proliferation than commercial red cabbage pigments; the sorghum extract inhibited 50% of HT-29 cell proliferation at less than 200 μg/ mL, whereas red cabbage extract was ineffective even at 800 μg/mL.

Table IV. *In vitro* inhibition of human leukemia (HL-60) and liver (HepG2) cancer cell proliferation by 3-deoxyanthocyanidins, luteolinidin and apigeninidin, compared to their anthocyanidin analogs, cyanidin and pelargonidin. Data transformed from Shih et al (*8*)

Cell line	*Concentration*	*Luteolinidin*	*Cyanidin*	*Apigeninidin*	*Pelargonidin*
HL-60	100 μM	55	85	70	90
	200 μM	07	78	35	78
HepG2	100 μM	65	100	62	100
	200 μM	53	100	55	100

Percent cancer cell proliferation relative to untreated control at two concentrations of the compounds shown.

Other evidence we have gathered indicate that tannin sorghums also show much higher potency than grape seed extract at inhibiting the HT-29 cancer cell growth (Figure 8). In fact Awika et al (*60*) reported that tannin sorghum extracts were more potent than black, red or white sorghum extracts at inhibiting HT-29 as well as OE33 esophageal adenocarcinoma cell proliferation. The tannin sorghums inhibited 50% of HT-29 and OE33 cell proliferation at 38 – 65 μg/mL. These values are much lower than those reported for most natural extracts (typically above 1000 μg/mL). The evidence clearly suggests that the sorghum compounds have much higher potential to suppress preformed cancer from rapid proliferation than their analogs found in other food sources, and may be relevant for dietary applications at low use levels.

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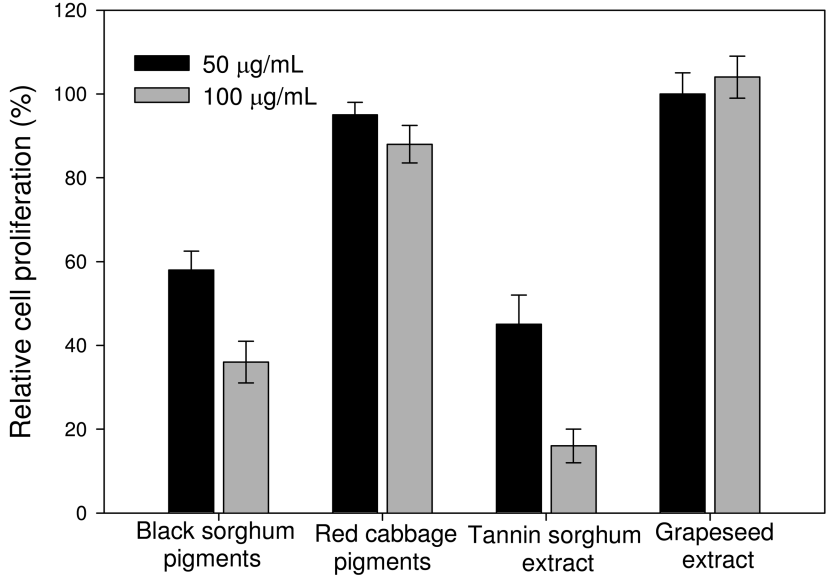

Figure 8. Comparative inhibition of in vitro HT-29 colon cancer cell proliferation by sorghum pigment and tannin extracts, and commercial red cabbage and grape seed extracts. Cells (3000/well) were incubated with extracts for 48 hrs before assay, MTT method was used. Error bars represent ± sd based on three separate experiments; unpublished data.

Cardiovascular Health

Whole grain consumption is well known to correlate with reduced incidences of cardiovascular disease (CVD). For example, a large 10 year study (*80*) found that the level of whole grain intake inversely correlated with the risk for coronary heart disease among women, with the highest level of intake associated with 50% reduction of risk. The authors also concluded that the level of CVD risk reduction could not be fully explained by the contribution of whole grain dietary fiber, folate, vitamin B-6, and vitamin E, which implies other components may be important. Whole grain is a good source of dietary fiber, minerals, vitamins, antioxidant and other beneficial compounds. Pinpointing the active components of whole grain that benefit cardiovascular health is thus a difficult task. However, some data suggests that the bran components and not the germ (which is rich in minerals and vitamins) contribute to the benefits of whole grain consumption (*81*). The authors reported that whole grain consumption in general reduced coronary heart disease risk by 18% among men, whereas added bran in diet reduced the risk by 30%; added germ did not produce any benefit. We are not aware of any studies that compare benefit of different sources of whole grain on CVD risk.

Uncontrolled free radical challenge and chronic inflammation in the body are known important causes of CVD. It is thus not surprising that many compounds the act as antioxidants and anti-inflammatory agents also tend to benefit cardiovascular health. For example, low density lipoprotein (LDL) oxidation

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may lead to artery wall damage and eventually atherosclerosis by increasing inflammation in the blood vessel wall. Dietary antioxidants are believed to play an important role in keeping LDL oxidation in check. Numerous epidemiological studies have shown that consumption of products high in flavonoids, like red wine and tea correlate with reduced risk of CVD (*82*, *83*). These effects are largely attributed to the tannins and other polyphenols in these food products. Even though there is no epidemiological data on tannin sorghum consumption and CVD risk in humans, it is likely that sorghum tannins may have similar benefits. In addition, the monomeric 3-deoxyflavonoids are also likely to contribute to cardiovascular health, given their demonstrated high antioxidant and anti-inflammatory properties. With the growing risk for obesity and CVD in the regions where sorghum consumption is significant, a study in this regard is highly warranted.

Concluding Remarks

Sorghum contains a diverse array of flavonoid compounds, most of which are not typically found in other cereal grains. The high levels of compounds like 3-deoxyanthocyanins, pro(3-deoxy)anthocyanidins, flavones, and flavanones in certain sorghum varieties is especially of interest both from a commercial and health perspective. Given that the types and levels of the flavonoids in sorghum are controlled by a set of well documented genes; it is possible to breed for sorghum varieties that accumulate specific compounds at levels that can be exploited for targeted food/health applications to produce desired benefits. For example, the levels of 3-deoxyanthocyanin pigments in black sorghum bran as well as purples sorghum plant glumes and other plant tissues are high enough to warrant commercial interest as natural food colorants. The fact that these sorghum pigments are relatively stable compared to other natural colorants is an added advantage. The high levels of flavanones in lemon yellow sorghum varieties, and proanthocyanidins in type III sorghum could provide an economical way to obtain these well known bioactive compounds for nutritional applications.

Evidence suggests that the unusual compounds in sorghum and the high levels at which they are present in the grain produce specific health benefits that are not observed for other grains like corn, rice, or wheat. The epidemiological evidence that indicate sorghum consumption may be more chemoprotective, especially against gastrointestinal cancer, is an interesting example that warrants additional investigations. The laboratory evidence based on cell and animal models seem to support the epidemiological evidence for higher efficacy of sorghum relative to other grains. The fact that sorghum 3-deoxyanthocyanins are more cytotoxic to cancer cells; that O-methylated 3-deoxyanthocyanins are powerful phase II detoxifying enzyme inducers; that sorghum brans elicit anti-inflammatory response not observed for other cereal brans *in vitro* and *in vivo*; that sorghum phenolic extracts are more powerful antioxidants than other grains, or fruits and vegetables, among other, all warrant a closer look at sorghum as a commodity that can significantly contribute to chronic disease prevention.

Important questions remain as to how sorghum components contribute to the various observed superior benefits. Whether it is simply related to

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better membrane permeability of, say, the 3-deoxyanthocyanins relative to anthocyanins, or specific structural conformations that fit better with target cell receptors, etc, are unknown. The biochemical basis for these observations must be unraveled. Other broader questions will need to be addressed: What levels of sorghum consumption would produce desired benefits in humans? (This would be particularly interesting from a long term disease prevention perspective, rather than short term therapeutic-type studies.) How would the different sorghum varieties with varying flavonoid composition benefit specific health outcomes? How can food processing be optimized to maximize the beneficial effects of sorghum flavonoids?

The fact that sorghum is currently mostly consumed in the developing world has been somewhat a setback in terms of stimulating widespread research into the specific health benefits of the grain, especially in relation to chronic disease. However, with the growing number of 'Western' health problems like obesity and cardiovascular disease in developing countries, an opportunity to gain better insight into sorghum consumption and disease prevention is upon us. An additional question of interest is whether sorghum flavonoids could contribute to strengthening of the immune system; this would be particularly important given the scale of HIV in Africa, which is also the largest sorghum consuming region.

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Editors' Biographies

Joseph M. Awika

Joseph Awika is on faculty in the Soil & Crop Science, and Nutrition and Food Science Departments at Texas A&M University, College Station, TX, where he teaches and conducts research. His research focuses on functional and processing aspects of cereals and legumes, especially on factors that influence health attributes of these crops. He is particularly interested in grain flavonoids and how they interact with other reactive molecules in foods to influence food quality and bioactive properties. Prior to joining Texas A&M in 2008, he was on Faculty at University of Missouri-Columbia, MO, and Arkansas State University, Jonesboro, AR. Joseph currently serves as associate editor for the Journal of Science of Food and Agriculture.

Vieno Piironen

Vieno Piironen is a professor in Food Chemistry at the Department of Food and Environmental Sciences, University of Helsinki. She investigates bioactive compounds of the dietary fibre complex in cereals focusing especially on vitamins and phytosterols. Recent collaboration projects have focused on enhancing vitamin contents in cereal-based foods by plant breeding, fractionation of the materials and bioprocessing. Natural fortification using microbial *in situ* synthesis of folate and vitamin B12 is of special interest. In addition, oxidation reactions of lipids and reactions leading to vitamin losses are among her research interests.

Scott Bean

Scott Bean received his Ph.D. in Grain Science from Kansas State University in 2001 and joined the USDA-ARS as a research chemist in 2001. His research has included developing improved methods for characterizing properties of cereal proteins as well as investigating the role of proteins in cereal food quality. Scott is currently researching how the proteins of sorghum are cross-linked together and how this cross-linking impacts the functional and nutritional properties of sorghum. His group also conducts research on the production of gluten free foods from sorghum. Scott is also an adjunct assistant professor in the Department of Agronomy at Kansas State University and serves on the advisory board for the Celiac Sprue Assocation and the Editorial board for the Journal of Cereal Science. Scott has authored or coauthored over 90 peer reviewed publications and several book chapters.

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