Identification of genetic, environmental and technologic factors associated to the variability of vitamins in common wheat and wheat based food products

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Thèse

Identification of genetic, environmental and technologic factors associated to the variability of vitamins in common wheat and wheat based food products

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Résumé

Le blé est la seconde céréale la plus cultivée dans le monde et constitue un apport majeur de l'alimentation quotidienne. L'effort consentie à continuellement améliorer les qualités meunière et boulangère du blé tendre, s'est fait au détriment du caractère nutritionnel du grain. Ainsi la plupart des produits industriels dérivés des grains de blé sont produits à partir de farines blanches raffinées qui ne contiennent ni le germe ni les sons. Cependant, dans ces différents tissus qui sont éliminés et qui servent essentiellement à nourrir les animaux, se concentrent les principaux micronutriments tels que les vitamines, les minéraux, les fibres et des substances phytochimiques. Les différentes enquêtes épidémiologiques ont bien mis en évidence les conséquences négatives de la déplétion en micronutriments des produits céréaliers raffinés. Dans l'objectif d'une alimentation plus saine voir même préventive, la consommation d'aliments enrichis en micronutriments naturellement présents dans le grain de blé tendre semble être une démarche efficace. Dans cette optique, ce travail de thèse a permis de consolider et d'accroitre les connaissances concernant les voies d'amélioration des teneurs en vitamines des grains de blés tendres ainsi que des produits industriels qui en sont dérivés.

En premier, nous nous sommes intéressés au développement d'une méthode simple et rapide basée sur la spectrométrie de masse couplée à la chromatographie liquide pour la détermination simultanée de sept vitamines hydrosolubles dans divers matériels végétaux. Les vitamines présentes dans les différents matériels végétaux furent séparées en moins de 15 min grâce à l'utilisation d'une colonne C18 en phase inverse, et analysées en mode ElectroSpray positif et MRM. La réponse pour toutes les vitamines a été linéaire sur l'ensemble des concentrations étudiées (0.05 to 9 µg/mL) avec des coefficients de corrélation compris entre 0.991 et 1. Les limites de quantification de la méthode analytique ont été évaluées entre 0.09 et 3.5 µg/g. Les précisions intra-journalière et inter-journalière étaient satisfaisantes.

La deuxième partie de nos travaux a concerné l'impact des procédés de transformation du grain (production d'une nouvelle fraction de mouture et grillage) sur la teneur en vitamines. Afin de réaliser cette objectif, la méthode développée a été appliquée pour l'analyse simultanée des concentrations en vitamines hydrosolubles contenues dans différentes farines semi-complètes ainsi que dans les pâtons, pains et pains grillés qui en sont dérivés. En parallèle, les concentrations endogènes des vitamines E, de la Lutéine et du β-sitostérol ont également été évaluées dans le même matériel. Nous avons mis en évidence que les concentrations en acide nicotinique, pyridoxale, pyridoxine et acide pantothénique étaient significativement plus élevées dans les gros sons que dans les autres fractions de moutures, alors que les concentrations en β-sitostérol, lutéine, α-tocotriénol, α-tocophérol et thiamine (20.87 µg/g DM)étaient plus importantes dans la fraction de mouture enrichie. L'étape de grillage induit une augmentation significative en α-tocophérol (+216%), β-γ-tocophérol (+52%), α-tocotriénol (+83%), β-γ-tocotriénol (+32%), acide nicotinique (+55%), nicotinamide (+97%) et en pyridoxine (+77%). L'ensemble de ces résultats nous a permis de montrer qu'un enrichissement de farine blanche par la fraction de mouture dite enrichie pourrait potentiellement permettre d'accroître les produits qui en dérive en vitamine E. De plus le grillage pourrait libérer des composés bioactifs, augmentant ainsi leur biodisponibilité et la valeur nutritionnelle des pains.

Cette thèse a également montré l'influence de la variabilité génétique et environnementale sur les teneurs en vitamines d'une core collection de 195 accessions de blé tendre cultivés en deux lieux très différents. Les résultats obtenus indiquent qu'il existe une influence significative de l'effet génétique sur la variabilité des concentrations en nicotinamide, riboflavine et acide pantothénique pour les vitamines du groupe B et un impact significatif sur la teneur en des composés bioactifs liposolubles tels que l' α-tocotriénol, β-sitostérol et la lutéine. L'influence des facteurs du milieu de culture sur la variabilité des vitamines a également été significative, et il a été suggéré que les valeurs d'héritabilité en vitamines hydrosolubles et en molécule bioactives liposoluble des accessions de la core collection étudiée étaient très élevées. De plus, il semble que la sélection intensive n'ait pas diminué les concentrations en ces composés d'intérêts.

Enfin, des zones chromosomiques impliquées dans la régulation des teneurs en vitamines des grains de blé tendre ont été recensées. Ces nouvelles données génétiques pourraient aider les sélectionneurs à développer des nouveaux marqueurs moléculaires qui permettraient d'accélérer la sélection de variétés de blé tendre enrichies en vitamines.

Mots clefs :

Vitamines, Blé tendre, LC-MS/MS, Fractions mouture, Grillage, Core Collection, Association génétique.

Abstract

Wheat is the second largest crop cultivated around the world and constitutes a major part of the daily diet in Europe. During the course of improving the baking quality of wheat cultivar, most of the nutritional attributes have been underestimated. It is therefore unfortunate that most of wheat-based food products are mostly produced from refined white flour from which peripheral tissues (germ and envelopes) are removed. However, these tissues, which are eliminated and serve mainly for animal feeding, contain most of the vitamins, minerals, fiber and phytochemicals of the grain. It is becoming evident that many of the health benefits associated with the consumption of whole grain cereal products, relate to the enhanced intake of micronutrients, phytochemicals and dietary fiber. In the context of consuming wheat derived foods with enhanced nutritional value, as part of a healthy diet, this thesis provide results which strengthen the knowledge of vitamins accumulation in common wheat and in wheat-based food products.

Firstly, we have developed a simple and rapid method based on liquid chromatography tandem mass spectrometry (LC-MS/MS) for the simultaneous screening of seven water soluble vitamins in various wheat-based food materials. The vitamins present in the test materials were separated in less than 15 min by using a reverse-phase C18 column, and analyzed by positive ion electrospray selected reaction monitoring MS/MS. The MS response for all the vitamins was linear over the working range (0.05 to 9 µg/mL) with correlation coefficients ranging between 0.991 and 1. Limits of quantification in the different food materials ranged from 0.09 to 3.5 µg/g. Intra-day and inter-day precision was found satisfactory.

The second part of our research, have focused on monitoring the levels of vitamins upon the wheat-based foods processing operations, such as production of new wheat milling fraction (consisting in enriched fraction) and breadmaking toasted bread. In order to achieve this goal, the developed method was applied for the simultaneous analysis of the water-soluble vitamin natural content of different semi-coarse wheat flours and in their corresponding baking products. In addition the vitamin E, Lutein and β-sitosterol natural content was also measured in the same materials. It was shown that the concentration of nicotinic acid, pyridoxal, pyridoxine, pantothenic acid were significantly higher in the coarse bran than in the other milling fractions, while the concentration of β-sitosterol, lutein, α-tocotrienol, α-tocopherol and thiamin (20.87 µg/g DM) were the highest in the enriched fraction. The toasting step induced a significant increased of α-tocopherol (+216%), β-γ-tocopherol (+52%), α-tocotrienol (+83%), β-γ-tocotrienol (+32%), nicotinic acid (+55%), nicotinamide (+97%) and of pyridoxine (+77%). Furthermore, it was demonstrated that the enriched fraction could be a functional ingredient in order to enrich wheat-based products in fat soluble vitamins and that the toasting process could release bound bioactive compounds and led to enhance the nutritional quality of bread.

This thesis also addressed the influence of genetic and environmental variability on the nutritional status of bread wheat varieties of a large international bread wheat core collection grown in two contrasting growing location. The results presented indicate that genotype had a significant impact on the variations of nicotinamide, riboflavin and pantothenic acid contents for the B-vitamins and significant impact on the variations of α-tocotrienol, β-sitosterol and lutein contents for the fat-

soluble bioactive compounds. In addition, the growing environment may significantly affect the vitamin compositions and, it was suggested that within the core collection used in this study, the variation in B and lipid-soluble bioactive componds was highly heritable. The evaluation of the relationship between the contents of vitamins and the release dates or geographical origins of wheat accessions in the core collection, has led to the conclusion that the contents of vitamins have not decreased with modern plant breeding.

Finally, relevant genomic regions involved in the genetic control of wheat vitamins composition have been reported. Thus, the current analyses provides innovative and relevant genetic data to develop new molecular markers to accelerate genetic gain for vitamin content in bread wheat.

Keywords :

Vitamins, Bread wheat, LC-MS/MS,Wheat mill fractions, Toasting, Core Collection, Association mapping.

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«Que l'alimentation soit ton premier médicament»…

Hippocrate, 2500 years ago…

In Memory of My Beloved Father

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Introduction

In its 8000-years history, wheat continues to be a major food grain crop consumed by humans . World wheat production, now averaging nearly 713 million tons annually, still needs to be improved to keep abreast of population growth. Wheat research in the twentieth century has been monumental in providing a basic understanding of this food crop in terms of origin, culture, genetics, food quality aspects and how to improve its productivity. Beside the considerable efforts made to increase yield potential , and to improve resistance to both biotic and abiotic stresses under widely varying environments, a lot of researches have also been focusing in changing the technological quality of wheat grain by developing it for industrial uses. Thus, in France near 80% of new wheat cultivars are now registered as superior bread-making wheat quality class. During the courses of improving the baking quality of wheat, most of the nutritional attributes have been underestimated. It is therefore unfortunate that most of the wheat-based food products are produced from refined white flour from which peripheral tissues (germs and envelopes) are removed. However, these tissues which are eliminated and serve mainly for animal feeding, contain most of the vitamins, minerals, fiber and phytochemicals of the grain. Nowadays, numerous epidemiological surveys have shown a significant and positive association between whole-grain cereal consumption and the prevention of several chronic diseases. It is becoming evident that many of the health benefits associated with the consumption of whole grain cereal products, relate to the enhanced intake of micronutrients, phytochemicals and dietary fiber. In order to promote the health benefits of the consumption of whole-grain cereal, new programs have emerged in France with the PNNS (Programme National Nutrition Santé) and in Europe with the HEALTHGRAIN program. In this context of consuming wheat derived foods with enhanced nutritional value, as part of a healthy diet, this thesis provide results which strengthen the knowledge of vitamins accumulation in common wheat and in wheat-based food products. Targeted metabolomic approach, as well as technological , agronomic and genetic aspects have been considered within this manuscript to further knowledge for mapping regions responsible for the variation in vitamin contents, for explaining the variability of vitamins upon the industrial wheat products processing operations and for exploring the extent of variation in vitamins composition in bread wheat varieties from a core collection representative of the world's wheat diversity. This manuscript is organized in eight chapters.

- The first chapter is devoted to the literature review. Apart from general subject background, the literature review has focused on three themes : 1) Genetics, wheat genetic improvement and wheat grain quality, 2) Development, structure and constituents of wheat grain, and 3) The different approaches to improve wheat vitamin contents.
- The second chapter describes the research aims of this thesis and the third one give an overview of the different materials and methods employed.
- The works described in chapter four are the subject for the first publication entitled **Development of a LC-MS/MS method for the simultaneous determination of 7 water-soluble vitamins in processing semi-coarse wheat flour products.**
- The fifth chapter is consecrated to the study of the impact of wheat processing industry on the variation of vitamins. The results of this study are discussed **Change in B and Fat-soluble vitamin contents in industrial milling fractions and during toasted bread production.**

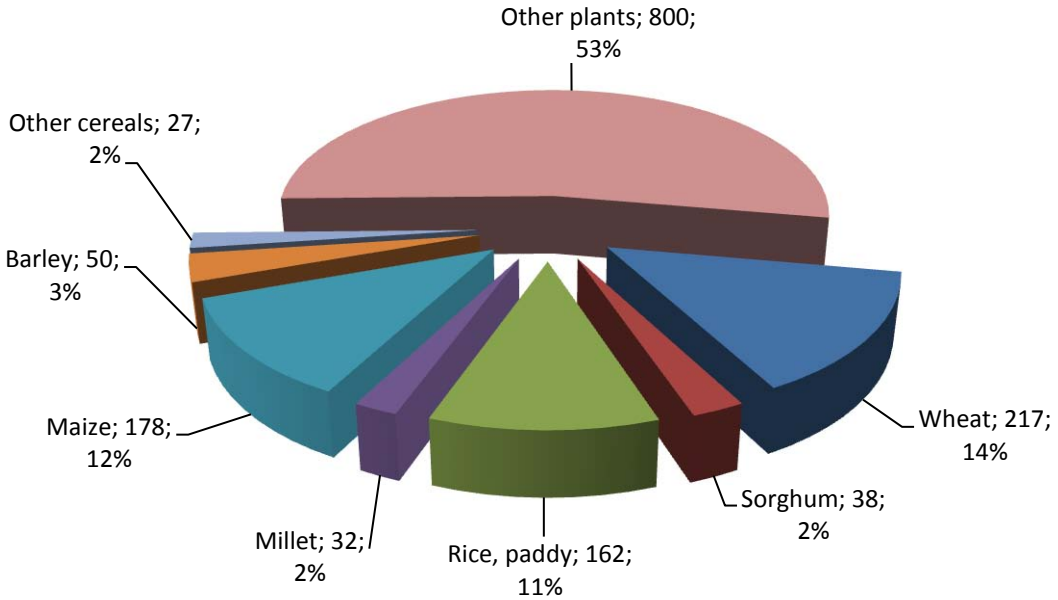
- The influences of genetic and environmental variability on the vitamins status of bread wheat varieties will be discussed in chapter six.
- The results of the association study of wheat grain vitamins composition will be discussed in chapter seven.
- This thesis will end with the last chapter which highlights the main results obtained and proposes scientific research perspectives.

Literature review

1. Position of Europe and France in the world's wheat production

Wheat, maize and rice dominate world's grain production. The importance of cereals appears easy to explain: relative to other grain crops, yields are both high and stable; compared to root and tuber crops, cereal grain is easy to grow, transport and store (Gooding, 2009). Wheat is among the oldest and most extensively grown of all grain crops. Western agriculture is thought to have started around 10,000 BC somewhere along the Fertile Crescent in the Near East (Araus et al., 2007). Neolithic agricultural practices, probably including growing in natural wet soils, seem to have produced relatively high wheat production yield, which most likely enabled the global transition from gathering to cultivation to take place (Araus et al., 2007). Nowadays, world has plant production area of around 1500 million hectares (FAOSTAT data, 2014), accessed via the website at ww.fao.org). From figure 1, it can be seen that growers are producing cereals on 47% of sowing area in the world. Maize, rice and wheat are sharing 37% from entire sowing area. In 2012, the world harvested area of wheat was 217 million hectares, making it the first most-sowed cereal before maize (178 million Ha) and rice (162 million Ha) (figure 1). Thew, proportion of remaining cereals in comparison to entire sowing area of the world is 2 %. It has to be mentioned that nearly half of the global wheat area is in developing countries. World economy role of wheat production is significant in terms of cultivated land and food supply, feeding and commerce.

Figure 1. Division of sowing area in the world in 2012 (in millions hectares and percentage) (Source: FAO, 2013)



Wheat is the second most-produced cereal in 2013 with 713 million tons after maize (1020 million tons) and just before rice with 646 million tons (Source: FAO, 2013). Table 1, shows the tendency of world's wheat production from 1993 to 2013, there are minimal differences in case of harvested area during this 20-years period. There is a slight increase of crop yield comparison since 1993. Average 2.5 tons wheat was produced on one hectare crop land in the world in the first half of 1990's, however this value was about 3.3 tons in 2013.The reason for this appears to be related to the result of a slow and successive increase of the average yield as during this period no development of the sowing area was noticed. In France, the last twenty years have shown an increase of planting area (from 4.51 to 5.32 million hectares) (Table 1) and of wheat production (from 292 to 386 million tons), reflecting a higher yield.

Table 1. World and France 's wheat production from 1993 to 2013 (Source : FAO, 2013).

Years	**Area harvested** (*million hectares*)		**Production** (*million tons*)		**Average yield** (*t/ha*)	
Country	***World***	***France***	***World***	***France***	***World***	***France***
1993	222	4.51	564	29.21	2.53	6.47
1994	215	4.57	527	30.50	2.45	6.67
1995	216	4.75	542	30.88	2.51	6.51
1996	226	5.04	585	35.95	2.58	7.13
1997	226	5.11	613	33.85	2.71	6.62
1998	220	5.23	593	39.81	2.70	7.61
1999	213	5.12	587	37.05	2.75	7.24
2000	215	5.25	585	37.35	2.72	7.12
2001	214	4.77	589	31.54	2.75	6.62
2002	213	5.23	574	38.94	2.69	7.45
2003	207	4.88	560	30.47	2.70	6.25
2004	216	5.24	632	39.69	2.92	7.58
2005	219	5.28	626	36.89	2.85	6.99
2006	211	5.25	602	35.36	2.85	6.74
2007	216	5.24	612	32.76	2.83	6.25
2008	222	5.49	683	39.01	3.07	7.10
2009	224	5.15	686	38.33	3.06	7.45
2010	216	5.93	649	38.21	2.99	6.44
2011	220	5.83	699	35.99	3.18	6.18
2012	217	5.30	671	40.30	3.09	7.60
2013	218	5.32	713	38.61	3.26	7.25

In general, when comparing the average wheat yield around the world, it appears that France occupies the first place. Indeed, in 2013, in term of rank yield, France was second just behind Germany but far in front of China, USA, India and Russian federation (Figure 2). Figure 3 shows the hierarchy based on produced amount of wheat by European Union (EU) composed of 28 countries in 2013. During this year, the EU produced 143 million tons of

wheat, with 86 % of this amount being produced by the ten first ranking countries. With this production, the biggest world's wheat producer in 2013 was EU, followed by China, India, USA and Russian federation (Figure 4). In general, France is the biggest wheat-producer in the EU. In the hierarchy based on wheat production, France and Germany take place within the top 10 list of the world's wheat producer countries. There are not so many differences between the productions of the countries in the 5th to 10th places of the list, than in case of the top three countries. In rank of leading wheat growers in the world, the countries are the same apart from minimum deviation between 2000 and 2013 (Istvan, 2011). However, in this given period, there was an example that two large wheat producing countries changed their place in the rank during certain years. In fact, between 2003 and 2008, the USA were the third largest wheat producing country in the world while in 2009 and 2011 Russia gained the third place of this contest.

Figure 2. Wheat production yield in 10 major producing countries in 2013 (Source: FAO, 2013)

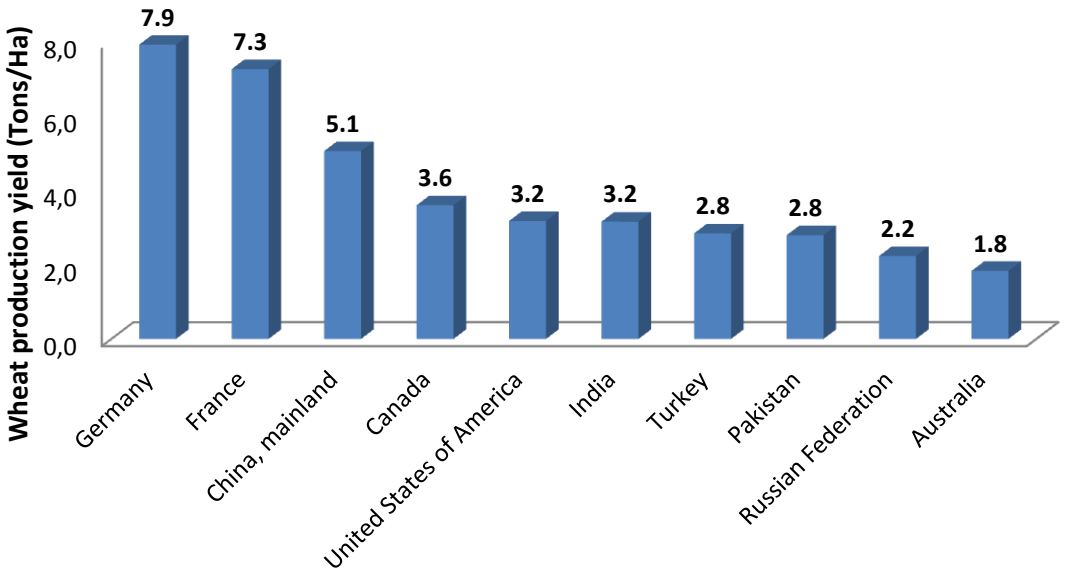


Figure 3. The EU's 28 countries ranked in terms of wheat production in 2013 (Source: FAO, 2013)

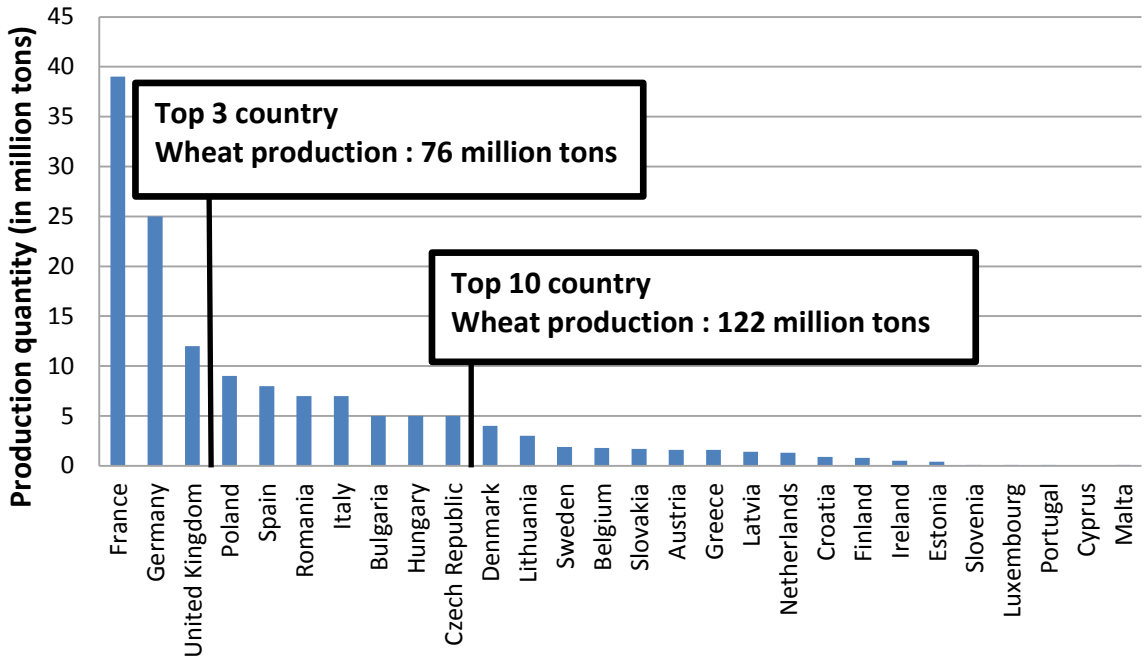
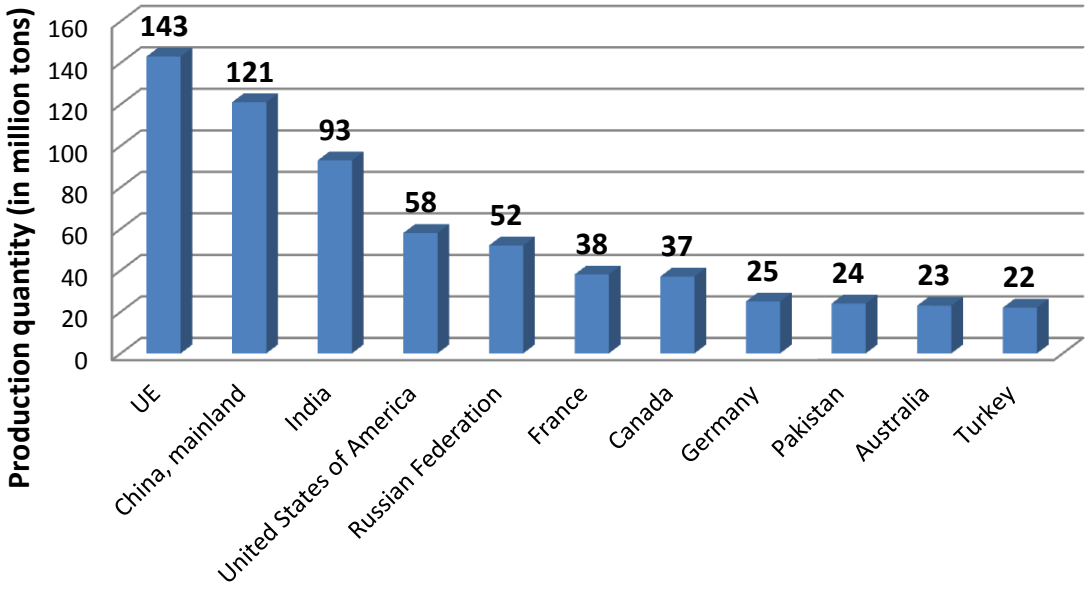


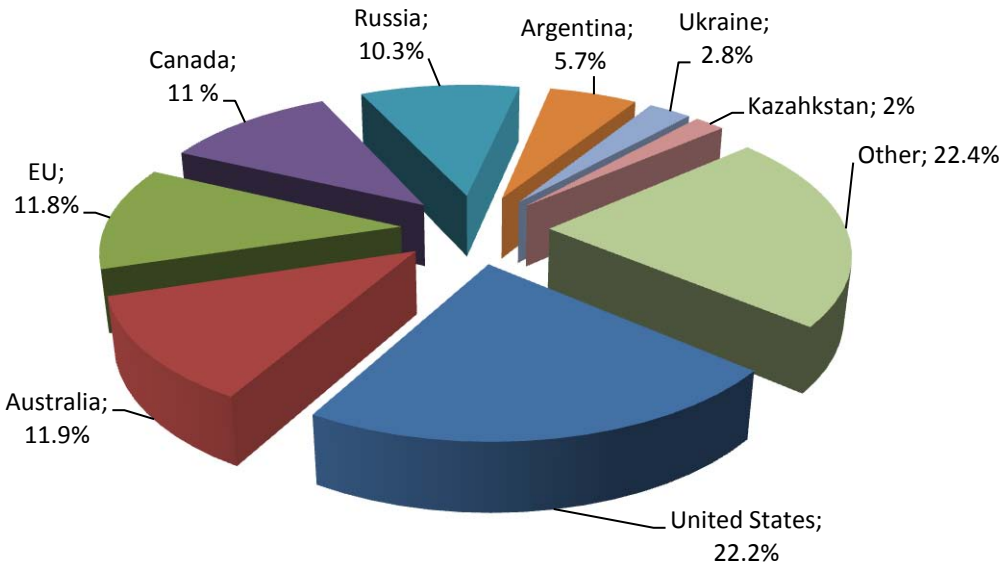
Figure 4. Production quantity of the top world's wheat producer in 2013 (Source: FAO, 2013)



Global wheat in 2014 is forecast at 718.5 million tons, a marginal increase compared to 2013 record output (Source: FAO, 2014). The 2014 aggregate output of wheat in European Union is estimated at about 147 million tons, which represents an improvement of 2.4 percent up

from 2013 (FAO, report October 2014). Exports of wheat are dominated in 2011 by five exporters: the USA, Canada, Australia, Russia and the EU (Figure 5). Two thirds of all exports came from these five countries. However, in the last decade, world trade in wheat has shown a different pattern, as the five major exporters were USA, Canada, Argentina, Australia and the EU. The number one exporter continues to be the USA with export varying between 25.8 and 32 million tons during the last 10 years. Canada, Australia and the EU continue to fight for the second and third place. Argentina has decreased its exports of wheat during the last few years. Whereas exports in 2001 were 10.1 million tons, they barely reached 8.4 million tons in 2011. During the same time period, export of wheat from Russia increased by 35% (Source: FAO, 2001-2011). Russia has long been the world's leading importer of wheat. Therefore, even if Russia has now joined the ranks of the leading wheat-exporting countries, one should keep in mind the influence of the continental climate. As demonstrated in 2004 (Source: FAO), adverse weather conditions turned Russia from an exporter into a net importer of wheat. Egypt and Brazil regularly compete as the world's leading importer of wheat, with Egypt being dependent on imported wheat for 60 percent of its supply of that's grain. The experience of the 2007-2008 crises in Egypt was still a perfect example of the important political dimension of the international grain trade. Even if all the major importing countries are developing countries, the EU has been the number-one importer of wheat. It happened in 2002, when the EU-25 imported 13.1 million tons of wheat because of the huge supply of very competitively priced from the Ukraine and Russia. The EU reacted to this and implemented tariff rate quotas for wheat from these countries which limit the import volume to 3 million tons.

Figure 5. World wheat export (in percentage) (Source: FAO, 2011[a]).[a]: Value from 2013 was not available on the FAO website



The major factors used to distinguish wheat in trade are the hardness or softness of the grain, winter or spring habit, red or white bran color, and protein contents (Cracknell and Williams, 2004). Generally, wheat can be closely matched to many different end uses according to their grain hardness and protein content.

2. Wheat Consumption and end use.

Two thirds of the wheat production (Table 2) is used directly in products for human consumption and the rest is used as an ingredient in compound feedstuffs, starch production and as a feed stock in ethanol production. Since the beginning of the 21th century, wheat production and consumption have increased considerably, with an exception for the year 2006/2007 as the result of the drought in major producing countries.

Table 2. Wheat world balance (million tons) (source: FAO, 2013)

	2006	2007	2008	2009	2010	2011
Production	602	612	683	686	649	699
Consumption:	621	618	649	655	646	681
Food	440	442	444	447	455	457
Feed	110	103	129	128	110	142
Other uses	70	72	77	79	81	81

Wheat type and Classes

Common wheat (*Triticum. aestivum*) which is better known as hard wheat or soft wheat, depending on grain hardness, represents more than 90% of total wheat production. The flour milling industry is the main consumer of common wheat. Wheat flour obtained from the milling of these grains is used in the baking and confectionary industries and for home cooking. Because of its quality, attributes, particularly its very hard endosperm and high yellow pigment concentration, durum wheat is used to produce semolina which is the main raw material of pasta making. Some durum wheat is also milled into flour or into coarse durum grain grits to respectively manufacture medium-dense breads or produce couscous. Many importing nations have specific requirement concerning the qualities of the wheat they would like to import. For this trading purpose, different categories have been introduced such as grain hardness (soft, medium or hard) and color (red, white and amber). These categories are also subdivided into classes based on growing habit (spring or winter). For example, wheat in the USA is divided into eight classes: hard red spring wheat, hard red winter wheat, soft red winter wheat, durum wheat, hard white wheat, soft white wheat, un-classed wheat and mixed wheat. In addition, hard red spring wheat, durum wheat and soft white wheat classes are further divided into subclasses (USDA, Grain inspection handbook, Wheat, 2014, accessed via the website at http://www. gipsa.usda.gov/fgis/handbook/grain-insp/grbook2/wheat.pdf). Another important criteria used in wheat trade is grain grading which assures that a particular wheat stock meets the required set standards customers.

Wheat like other cereals are graded based on test weight, moisture content, maximal percentage damaged and foreign materials. Grain protein content and alpha-amylase activity (enzymatic activity associated with the germination of the grain) are also frequently considered as grading factors in wheat trading. These factors are important in evaluating the end-uses of wheat and can be tested rapidly upon reception of the wheat stock. Grain lots presenting important levels of amylase-activity may be totally rejected as a food item because these lots may present degraded starch within the kernel which would have a detrimental impact on the baking potential of flour obtained with such grain. Grade-determining factors constitute an effective means for describing wheat for marketing purposes.

Wheat consumption

Despite its use in the form of baked good for human consumption, wheat is also used as an ingredient for feeding animals and poultry. In 2011, 142 million tons (21 % of worldwide production) (Table 2) were used to feed animals. This proportion is higher in industrialized countries: in EU-27, 41% of wheat production was used as feed (FAO, 2011). Feed wheat is often surplus to human requirements or low-quality wheat unsuitable for the human consumption (low test weight or damaged wheat) but wheat is also grown for feed purposes (Lalman et al., 2011). Indeed, wheat grain has long been recognized as an excellent energy feed resource for livestock. However, the inclusion of wheat grain in feed depends on the relative market prices of the major feed grains. During periods when maize, barley or sorghum are expensive or when wheat market is depressed, wheat can be used as an economical feed source for beef cattle (Lalman et al., 2011). In recent years, the biofuels industry has been using wheat as the primary feedstock for ethanol production. The wheat used is generally downgraded wheat damaged by frost, disease or rains. According to Statista estimates, approximately 9 million tons of wheat were used for ethanol production in 2014, double the amount used in 2010 (Statista, 2014, accessed via the website at http://www.statista.com/statistics/202229/wheat-for-eu-ethanol-production-from-2010).

Currently, among different cereal crops, ethanol production from maize and rice has the highest yields (table 3). In the UE, wheat is the main feedstock for bioethanol plants whereas in the US the primary feedstock is maize.

Table 3. Bioethanol yield production from different cereal crops (Source: FAO, 2008).

Feedstock cereal	Cereal yield ton/ha	Conversion kg of ethanol /1ton	Biofuel yield ton/ha
Maize	4.9	400	1.960
Rice	4.2	430	1.806
Wheat	2.8	340	952
Sorghum	1.3	380	494

End-uses

Wheat is the leading source of vegetable protein in human food, having a higher protein content than other major cereals such maize or rice. Common wheat is used in bread

(leavened, flat and steamed), noodles, biscuits and cakes. Leavened breads which are popular in almost all parts of the world are made using hard to medium-hard wheat classes. Pastries, cookies or cake are made with soft wheat flours. The noodle is a staple food widely consumed in northern China and made from unleavened dough which is rolled flat and cut into a variety of shapes. The demand for instant noodles (fried and steam precooked) is increasing in the western hemisphere (World instant noodles association, 2014). Medium to medium-soft wheat grain hardness is preferred for Asian noodle (table 4). Durum wheat is used globally in alimentary pasta and regional foods (flat breads, couscous and bulgur) in North Africa and West Asia (Peña et al., 2008). Because of the awareness of a healthy lifestyle is increasing, more consumers in developed countries are willing for more nutritious wheat based food products with less fat or simple carbohydrate and enhanced benefic compounds such as vitamins or fiber. In order to assess this requirement, new wheat products like bread prepared with whole wheat flour, with multigrain flours or other functional ingredients have emerged from the bakery industry (Dewettinck et al., 2008).

Table 4. Wheat quality characteristics for various food types (From Peña, 2002)

Type	Grain Hardness	Grain protein (%)	Gluten (dough) strength type
Leavened Breads			
Pan-type, buns	Hard	>13	Strong-extensible
Hearth, French	Hard/Medium	11-14	Medium-extensible
Steamed	Hard/Soft	11-13	Medium/Weak
Unleavened (flat) breads			
Arabic	Hard/medium	12-14	Medium-extensible
Chapatti, tortilla	Medium	11-13	Medium-extensible
Crackers	Medium/Soft	11-13	Medium
Noodles			
Yellow alkaline	Medium	11-13	Medium/Strong
White	Medium/Soft	10-12	Medium
Cookies, cakes, pastries	Soft/Very soft	8-10	Weak/Weak-extensible

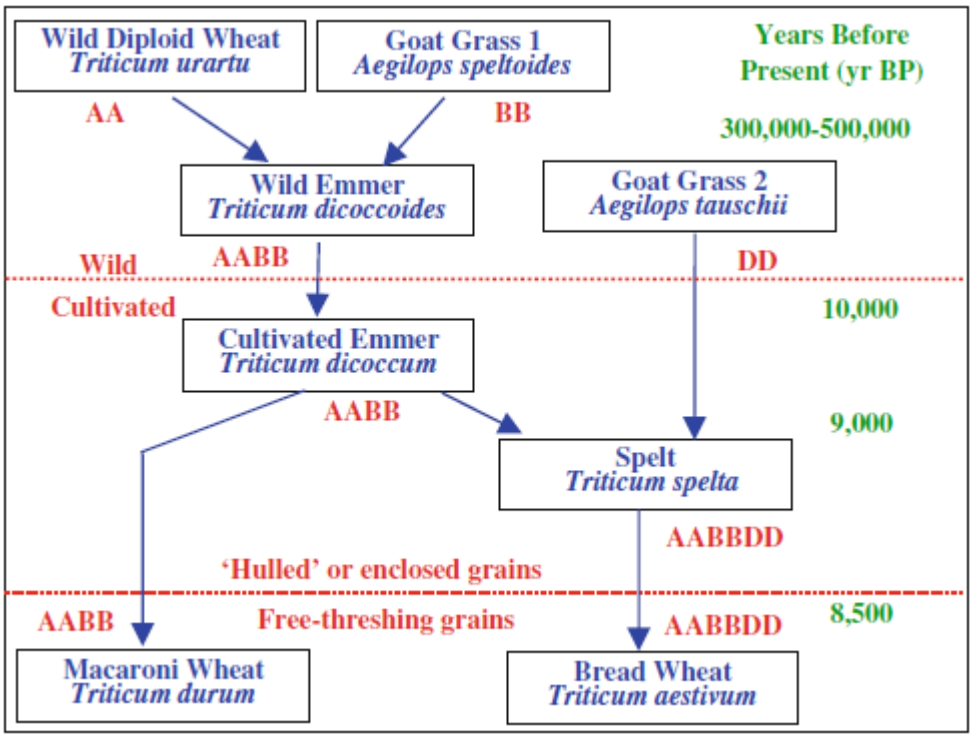
3. Genetics, wheat genetic improvement and wheat grain quality

3.1. Genetics and Wheat genetic improvement

Wheat genetics is more complicated than that of most other domesticated species. The genus name for wheat, *Triticum,* comes from the Latin, tero (I thresh). The current binomial name, *Triticum aestivum*, refers to hexaploid (2*n* = 6x = 42) bread wheat (genomes A, B, D), distinguishing it from tetraploid (2*n* = 4x = 28) durum wheat (*Triticum durum*) (genome A and B) which is used primarily for pasta production. The ancestral diploid wheat species are *T.monococcum*, *Aegilops speltoides*, and *T. tauschii* and a wild *Aegilops* species that is probably most closely related to the modern *Ae. Speltoides* (Khlestkina and Salina, 2001). Each of these have seven pairs of chromosomes (2*n* = 2x = 14). Tetraploids forms of current domesticated wheat are derived from a wild tetraploid progenitor, identified as the wild emmer *Triticum turgidum* ssp. Dicoccoides. This

species has an allotetraploid genome (AABB) resulting from spontaneous amphidiploidization between the diploid wild wheat Triticum urartu (AA genome, (Dvorak et al., 1998) and an unidentified diploid Aegilops species (BB genome). Around 9,000 BP a cultivated emmer (*T. dicoccum*, genome AABB) spontaneously hybridized with another goat grass (*Ae. Tauschii*, genome DD) to produce an early spelt (*T. spelta*, genome AABBDD) (Dvorak et al., 1998; Matsuoka and Nasuda, 2004). About 8,500 BP, natural mutation changed the ears of both emmer and spelt to a more easily threshed type that later evolved into the free-threshing ears of durum wheat (*T. durum*) and bread wheat (*T. aestivum*) (figure 6) (Peng et al., 2011). However, recent experimental evidence suggests that *T. spelta* is not the ancestral form of free-threshing common wheat (Dvorak et al, 2006). Apparently the sources of cultivated wheat ancestry are complicated by multiple factors including gene flow from wild cereals (Dvorak et al, 2011).

Figure 6. Wheat evolution (from http://www.newhallmill.org.uk/wht-evol.htm)



Complex traits such as yield, grain quality, disease, pest resistance and abiotic stress tolerance are controlled by specific genes that are distributed along wheat chromosomes. The A, B and *Ae.tauschii* (D) genomes have been estimated to contain approximately 28,000 (Choulet et al., 2010), 38,000 (Hernandez et al, 2012) and 36,371 (Massa et al., 2011) genes respectively. Recently Brenshley et al. (2012) estimated the number of genes in the hexaploid wheat genome to range between 94,000 and 96,000. In 2014, Choulet et al. produced the first reference sequence of the bread wheat chromosome 3B. Based on this success and methodology, the international wheat genome sequencing consortium (IWGSC), aims to finish sequencing the twenty other chromosomes within three years.

The major improvement during the last years, in wheat genomics and the sequencing through the development of specific bioinformatic tools and the used of thousands of molecular markers, are the achievement of continuous efforts initiated in the early 1950's. Development of biotechnology tools to accelerate the process of selecting parent lines that carry the desirable genetic materials for specific traits are essential, as it allows the appropriate selection at an early stage of selection process and thus avoid the need for ongoing propagation of undesirable lines. Simply inherited traits are selected early. Other traits such as yield and grain quality that involve many genes have traditionally been part of the mid- to late-generation selection schedule and are largely determined on the basis of actual phenotype.

Marker-assisted selection (MAS)

Plant molecular breeding is the applications of molecular biology or biotechnology to improve or develop new cultivars, which includes two major approaches, MAS and genetic transformation (Moose and Mumm, 2008). MAS is an indirect selection process whereby a marker (morphological, biochemical or DNA-based or molecular) linked to the trait of interest (disease resistance, productivity, abiotic stress tolerance and quality), is selected instead of the trait itself. Plant molecular breeding has advanced so rapidly that several types of molecular markers have been developed and used for decades. The very first genome map in plants was reported in maize (Helentjaris et al., 1986) using restriction fragment length polymorphism (RFLP). However, these markers utility has been hampered due to the requirement of radioactive isotope, time consuming of the technique and because of their inability to detect single base changes. With further advance of biotechnology, several types of polymerase chain reaction (PCR)-base markers were developed and used in plant breeding programs. These markers include, random amplification of polymorphic DNA (RAPD), sequence characterized amplified region (SCAR), cleaved amplified polymorphic sequences (CAPS), single sequence repeats (SSR), amplified fragment length polymorphisms (AFLP), direct amplification of length polymorphisms (DALP), single nucleotide polymorphisms (SNP) and diversity array technology (DarT). It has to be mentioned that the success of MAS require very tight linkages between markers and the trait.

Traits such as yield or baking quality are quantitative traits that depend on the cumulative actions of many genes and the environment. For breeders, it is important to detect in the selection process the lines which performed best for the traits of interest. Thus, it is assumed that those lines have a combination of alleles most favorable for the fullest expression of the quantitative traits. Another way to state this point is that the breeder would like to identify as early as possible those lines which contain those quantitative trait locus (QTL) alleles that contribute to a high value of the trait under selection. QTL analysis is predicated on looking for associations between the quantitative trait and the marker alleles segregating in the population (Kearsey and Farquhar, 1998). The other use of QTL is to identify candidate genes underlying a trait. Once a region of DNA has been identified as contributing to a phenotype, it can be sequenced. However, accurate QTL localization can be problematic as in the traditional QTL approach only recombination from the bi-parental cross are considered (Bordes et

al., 2010). Lately, association mapping based on the concept of linkage disequilibrium, has proven to be an efficient strategy to decipher the genetic basis of complex traits (Ingvarsson and Street, 2010). Using a core collection of 372 bread wheat accessions for association analysis of flour and dough quality traits, Bordes et al. (2010) have shown that out of 803 markers tested, 130 markers were associated with at least one trait studied. Association mapping was also successfully used by Plessis et al. (2013) to identify 74 loci associated with wheat grain storage protein content and composition and allometric scaling parameters of grain nitrogen allocation.

3.2. Wheat grain quality

In order to satisfy the increasing global demand and consumption of agricultural crops for food, particularly of wheat, researches on the improvement of yield potential of new wheat varieties have to be performed (Edgerton, 2009). Nevertheless, increasing yield potential without affecting negatively the quality of the grain is difficult, mainly because increases in grain yield are generally accompanied by a decrease in the grain's protein content, which is strongly associated with bread-making quality (Peña, 2002). Therefore, wheat breeders need to give grain quality aspects the same importance that they give to yield potential and diseases resistance.

Grain hardness

Endosperm texture (grain hardness) in wheat is the single most important and defining quality characteristic, as it facilitates wheat classification and affects milling, baking and end-use quality. Soft wheat kernels are easy to be fractured, which results in production of large number of intact starch granules and fine flour having less damaged starch. On the other hand, hard wheats require longer milling times and more milling energy, and produce a larger amount of damaged starch. Thus, hard wheat is more suitable for leavened breads because broken starch granules, that is damage starch, absorb more water, while flour of soft wheat is used for cookies, and pastries due to less protein and starch damage (table 4) (Morris and Rose, 1996). There are two main types of protein fractions which are associated tightly with starch granule, storage proteins (glutenins and gliadins) and starch granule- associated proteins (Pasha et al., 2010). The interaction between these proteins and starch influences the processing quality. The presence of a 15KD proteins, named friabilin and attached to the surface of the starch granule, has been strongly correlated with the qualitative level of endosperm hardness (Pasha et al., 2010). Friabilin is present in large amounts in soft wheat while hard wheat showed small amounts and durum wheat lacked it. Hardness or softness is inherited and controlled by a single locus referred to as Hardness (Ha), which comprises three genes Pin a, Pin b and Gsp-1, two additional degenerated copies of the Pin b gene, within a region of 80,000 bp (Chantret et al., 2005) on chromosome 5DS. Morris and Bhave. (2008) have reported 17 Pin a and 25 Pin b alleles in common wheat and related species. To summarize, absence or mutation of either or both of the two puroindolines, 'a' and 'b', in Triticum aestivum, result in harder grain texture. The most important methods used for measuring grain

hardness are particle size index (PSI), near infrared reflectance (NIR) hardness, pearling value, single kernel characterization system (SKCS), SDS-page, and PCR markers. NIR analysis of the particle size distribution of whole grain flour and analysis of the intact grain samples are particularly fast and useful on early generation screening (Peña, 2002).

Starch

Wheat milling can have detrimental effects on starch depending of the severity of the grinding and the hardness of the wheat, and the extent of the damage is of technological significance. Indeed, damaged starch content of flour is an important factor in determining baking quality. Some small amount of damaged starch is beneficial for the increase in baking absorption, gassing power of the dough and thus the volume of bread loaves. However, excessive starch damage can accelerate α-amylase action, decline crumb grain and texture characteristics by over-hydrating the dough leading to inferior baking performance (Rogers et al., 1994). In soft wheat products, especially in cookies and cakes, high level of damaged starch is highly undesirable. Barrera et al. (2007) reported that there was a consistent loss in cookies quality as the damaged starch content increased. The complete profile of starch pasting properties in wheat can be evaluated using the Amylograph/ Viscograph and more recently the Rapid Visco Analyzer (RVA).The RVA is 5 to 10 times quicker than the other instrument and require fewer samples (2 to 3 g). Therefore, the pasting properties of wheat are now commonly studied using the RVA to observe changes in the viscosity of a starch system based on rheological principles (Zaidul et al., 2007).

Proteins

Protein content is a key specification for wheat since it is related to many processing properties, such as water absorption and gluten strength. Protein content is also related to its end-product processing potential. Bakers use protein content results to anticipate water absorption and dough development time for processes and products, because higher protein content usually requires more water and a longer mixing time to achieve optimum dough consistency. The level of protein (between 8 and 17 %) is strongly influenced by environmental and management factors whereas the quality of protein is linked to genetic composition and environmental interaction (Eagles et al., 2002). Protein levels are an estimation based on the amount of nitrogen present in the grain. Protein content can be accurately measured by chemical methods (Kjeldhal or Dumas) and NIRS method. Protein quality measurement includes determination of the actual amount of gluten, assessment of viscoelastic dough properties and molecular measurement of amino acid groups (Wesley et al., 2008). Application of the NIRS technique in wheat quality control is nowadays widely accepted (Pojic et al., 2012).

4. Wheat grain: Development, structure and constituents

4.1. Grain development

4.1.1. Growth Stages from Germination through Maturation

The ten major growth stages that the wheat plant progresses through during its life cycle are:

1. Germination
2. Seedling
3. Tillering
4. Stem elongation or Jointing
5. Booting
6. Heading
7. Flowering or Anthesis
8. Milk
9. Dough
10. Ripening

Maturation or development of the grain

Germination begins with the uptake of water (imbibition) by a wheat seed that has lost its postharvest dormancy. Adequate soil moisture and temperature are needed for this to occur. Plant development is resumed once the embryo is fully imbibed. With the resumption of growth, the radicle and coleoptiles emerge from the seed. The coleoptile (second leaf) penetrates the soil and results in emergence of the seedling , usually within 5 to 7 days after planting as observed in the USA (Paulsen, 1997), though can be much slower in southern regions of Australia. The first roots on the wheat seedling are known as primary or seminal roots and include the radical previously mentioned. The functions of roots are anchoring of the plant body to the ground and support it, as well as absorption of water and inorganic nutrients. The seedling stage begins with the appearance of the first leaf and ends with the emergence of the first tiller. Up to six seminal roots and three leave support the plant at this stage. The crown of the plant becomes noticeably distinct after the third leaf has emerged. Crown formation is soon followed by the appearance of tillers and development of a secondary or crown root system. The crown root system provides mechanical support to distribute the leaves efficiently and serves as conduits for resources (water, nutrient to photosynthesis) to perform these functions. Each tiller that is produced represents the potential for a wheat plant to develop an additional stem complete with its own leaves, roots and head. A major change in the development of the wheat plant occurs at the end of the tillering stage. Jointing, the development of nodes and internodes that form the stem of the wheat plant, begins when growth of the tillers is complete. Indeed this phase marks the change from vegetative growth to reproductive growth. The stem elongation or jointing stage comes to an end with the appearance of the last (flag) leaf. The developing head

within the sheath of the flag leaf becomes visibly enlarged during the booting stage. The booting stage ends when the first awns emerge from the flag leaf sheath and the head starts to force the sheath open. At the heading stage, the spike emerges from the boot. Within 1 to 7 days after heading, the flowering stage and pollination occur and the grain begins filling (Paulsen, 1997). Wheat is classified as a long-day plant because it will flower only when days are long and night, the important times, are short. The grain begins growing immediately after fertilization and reaches its maximum size (not weight) within about two weeks. Grain development stages are determined by the hardness or the consistency of the endosperm of the new kernel. The developing endosperm starts as a milky fluid that increases in solid as the milky stage progresses. Maturation, or development of the grain, is divided into milk, soft dough, hard dough and physiological maturity, which is the stage when has reached its maximum weight. The kernel contains 30 to 35 percent moisture at physiological maturity (Paulsen, 1997). Ripening, the last stage, occurs as the grains loses moisture until it is ready to harvest.

4.1.2. Endosperm development

In cereal the endosperm is a persistent tissue and contains several specialized cell types. The bulk of the endosperm is composed of the starchy endosperm or internal endosperm cells, which accumulate starch and storage proteins. Adjacent to the pedicel is a transfer layer, specialized for nutrient uptake and transport from the maternal tissues to the developing endosperm. An epidermal layer, called the aleurone, covers the endosperm and during seed germination, secretes hydrolytic enzymes to digest the storage products in the starchy endosperm (Dupont and Altenbach, 2003). Understanding the formation of these different cell types will yield further insights into the suitability of immature wheat kernels for food processing, and the regulation techniques to obtain mature wheat flours that posses the desire properties (Katagiri et al., 2011).

The cytological events of wheat endosperm development (figure 7) are divided into four phases (Olsen, 2001).

-In the first few days after flowering (DAF), endosperm tissue is formed as a multinucleated syncytium.

-The cells division characterized by multiple rounds of mitotic division and cell wall formation will end when the entire volume of the endosperm is cellularized.

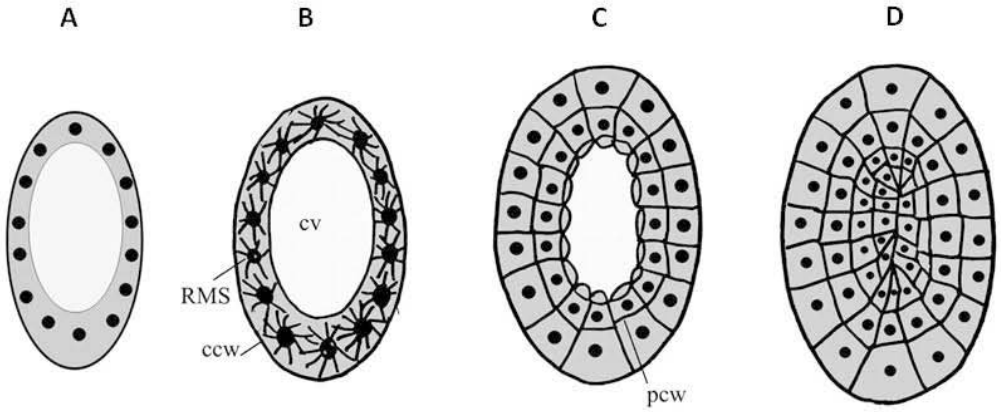
-During the third phase, large amounts of storage proteins, starch and other seed reserves accumulate.

-The late phase is a period of dehydration and seed maturation.

The development of the endosperm begins after the process of double fertilization or syngany, resulting in a diploid embryo and a triploid endosperm. Following fertilization of the central cell, the primary endosperm nucleus enters a period of

free nuclear division. Although the early endosperm is syncitial, the daughter nuclei of early divisions migrate to relatively invariant positions (Olsen, 2004). After a period of free nuclear division, the nuclei occupy the peripheral cytoplasm surrounding a large vacuole: it is the coenocytic endosperm (figure 7-A). Cellularization ensues through a process of free cell wall formation whereby unusual microtubule structures called adventitious phragmoplasts form at the boundaries between nucleo-cytoplasmic domains. The phragmoplast direct membrane vesicles containing cell wall constituents to the growing point of the cell walls. Beginning at the periphery and growing centripetally, cell walls grow between non-daughter nuclei, separating the nuclei into tube-like alveoli that remain open at their inner face. The next cell division occurs periclinally and accompanied by cytokinesis to yield a fully cellular peripheral layer and an alveolar internal layer (Becraft, 2001) . This process repeats until the entire volume of the endosperm is cellularized (Figure 7-B-D) (Olsen, 2001).

Figure 7. Cellularization of the endosperm coenocyte in Cereals.



(A) The complete endosperm coenocyte contains evenly spaced nuclei in the entire periphery of the coenocyt.

(B) Radial microtubule systems (rms) form on nuclear membranes in the cereal endosperm coenocyte. Ccw, central cell wall; cv, central vacuole.

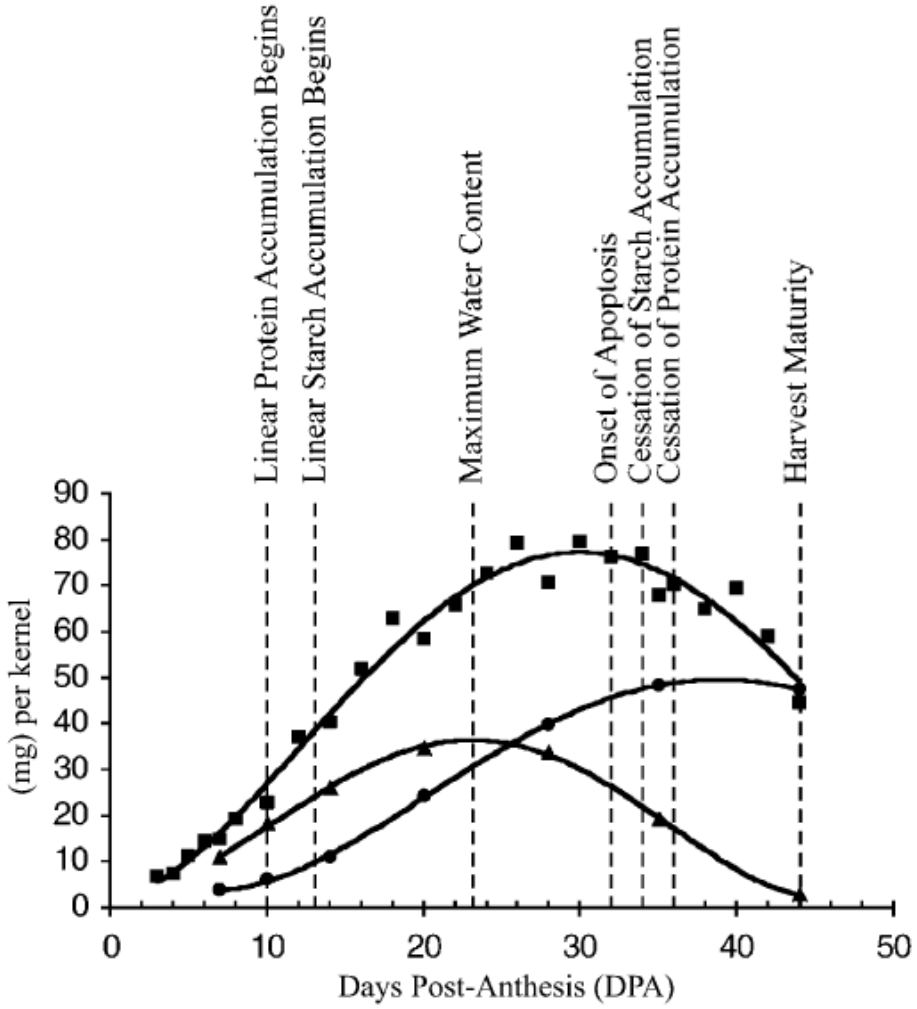
(C) Divisions of alveolar nuclei result in a periclinal cell wall (pcw) that separates the outer layer of cells from a new layer of alveoli.

(D) Repeated periclinal divisions in the innermost layer of alveoli continue until the endosperm is completely cellular. (Figure modified from Olsen, 2004)

The temporal pattern of grain development also can be described using physiologically criteria and tissue composition, which aimed to determine key phases according to transition points in the accumulation of water, total dry matter,

starch and proteins (figure 8) (Dupont and Altenbach, 2003). Cell expansion phase is a period in which water content increases and starches and proteins accumulate at a slow rate (Dupont and Altenbach, 2003). The importance of this earlier seed development might be explained by the close correlation between water mass per grain and/or the number of endosperm cells (Brockelhurst, 1977). The maximum amount of starch and protein that accumulate in each grain depend on the number of endosperm cells, determined early in grain fill, and the final size of the cells, which is influenced by water uptake, cell-wall extensibility and rate and duration of grain fill (Egli, 1998).

Figure 8. The temporal pattern of grain development described by transition points in the accumulation of starch, protein and water by the timing of the onset of apoptosis.■ , fresh weight;●, dry weight,▲ ,, water content. Figure modified from Altenbach et al., (2003)



Time and filling rate of grain wheat can largely vary depending of genotype and thermal condition of the cultivar (Yin et al., 2009). During grain fill stage, starch granules and proteins accumulate within endosperm cells (Singh et Jenner, 1982) until 15-20 days after anthesis, in a nearly linear way and the volume of grain

increase at reduced rate (Sofield et al., 1977). The next phase of development involves the differentiation of cells at the outermost periphery of the endosperm, giving rise to aleurone cells. Aleurone cells typically contain minimal amount of starch, instead accumulating oils and pigments and forming thick cell walls (Evers and Millar, 2002). Following the completion of the aleurone differentiation phase, cell expansion continues.

Cell expansion and water accumulation stop before the cessation of dry matter accumulation, starch and protein replace cell water, and the kernel begin to desiccate. Late in development, protein and starch deposition cease. Then, kernel dry weight usually reaches its maximum and kernel are said to be physiologically mature (figure 8) (Calderini et al., 2000). At approximately this time, some authors have proposed that the endosperm tissue undergoes a form of apoptosis or programed cell death (Young and Gallie, 1999) which is characterized by internucleosomal fragmentation of genomic DNA (Altenbach et al., 2003).

Finally, kernel desiccates rapidly until moisture levels that are safe for storage (less than 14.6% moisture), at which time they are ready for harvest (Altenbach et al., 2003).

4.2. General structure and major chemical components of wheat grain

4.2.1. Wheat Grain structure

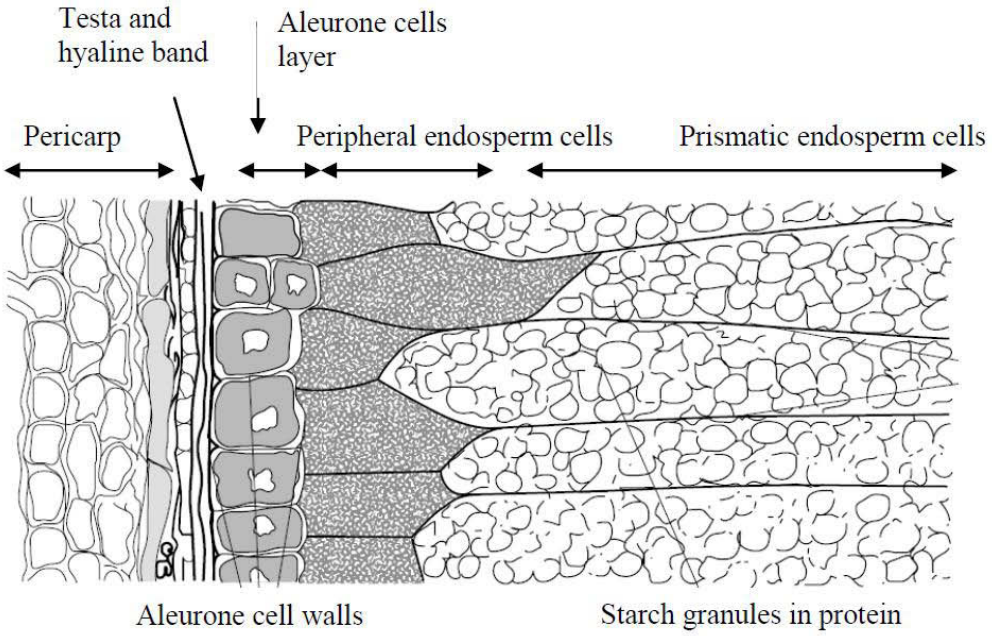
The wheat grain or wheat kernel is one seeded fruit called caryopsis. The wheat kernel has a longitudinal crease that extends almost to the center of the kernel. The existence of the crease creates technical milling challenges and requires special consideration in grinding. Some authors suggested the elimination of the longitudinal crease as a way of improvement for wheat. The dorsal side of the wheat grain can be oval, ovate or elliptical, with a cluster of or short brush hairs at the apex, and the oval or circular embryo at the bottom.

A wheat kernel is usually between 5 to 7 mm in length, 2.5 to 4 mm in width, 2.5 to 3.5 mm thick and weighs between 20 to 50 mg (Surget and Barron, 2005). Data related to the morphology of the wheat kernel vary in different research report. This variability is likely due to the different type and growing conditions of wheat analyzed, as well as the position of the seed on the ear. Recent research by Miller, (2008) on variation in single kernel hardness within the wheat spike reported that kernel hardness along the spike follows a natural gradient where the top and the bottom kernels are typically harder than those in the center of the spike. A close examination of kernel weight and diameters showed an opposite trend, with the top and the bottom having the smallest kernels (Miller, 2008). It was also shown it this study that 70% of kernels exist in the top and bottom portion of the spike, breeding programs that focus on developing this part of the spike, could bring improvements in grain yields and reduce variability.
Previous research has suggested that vascular effects are involved in the varied translocation of nutrients to the spike. It was suggested (Miller, 2008) that breeding

programs may target this, to ensure even levels of assimilate, which would likely promote uniformity within the spike.
The kernel is composed of three main anatomical parts: embryo/germ (3%), the outer layer (13-16% of the whole grain) and endosperm (80-85% of the whole grain) (Barron et al., 2007) (figure 10).

The Germ or Embryo, is a separate part of the grain, and is composed of two major parts, the embryonic axis (with rudimentary roots and shoots) and the scrutellum which is a transport organ of nutrition to the embryo during sprouting.
The bran (outer layers of wheat grain) is made up of several distinct cellulose-rich layers, which protect main part of the grain. The outermost layer, the pericarp (fruit coat), is made up of the outer pericarp, which includes the outer epidermis, hypodermis, thin-walled cells, and the inner pericarp, which includes intermediate-size cells, cross layers, and tube cells (inner epidermis). The latter do not form a continuous layer and are only recognizable in the mid-dorsal region of the mature kernel. The next layer inwards is the seed coat or testa which is strongly pigmented in red wheat and tightly bound to the nucellar epidermis (hyaline layer). The most outer tissues are rich in insoluble fiber. They are almost solely composed of thick cell walls formed of cellulose, cuticle materials and complex xylans with high arabinose to xylose ratios and substitution by ferulic acid dehydro-dimers, which act as cross-linkers between polymer chains (Hemery et al., 2007).
The endosperm represents the most abundant tissue found in the grain and it can be subdivided into two specific tissues. The aleurone layer has a special structure consisting of single layer (approximately 40-65 μm thick), of cubic shaped cells 37-65μm long x 25-75μm wide surrounded by thick cell walls (Surget and Barron, 2005). The aleurone layer is rich in proteins and contains the major part of the B vitamins and about half the total mineral content (Antoine et al., 2002; Pomeranz, 1988). During germination, the aleurone performs an important digestive function, secreting hydrolases to break down the starch and proteins stored in starchy endosperm cells.
The inner endosperm, i.e. the endosperm without the aleurone layer, is referred to as mealy or starchy endosperm. From outside to inside the seed, endosperm cells can be subdivided into three populations. Peripheral cells occurring as a single sub-aleurone layer and are approximately 60μm in diameter, prismatic cells 128-200μm long x 40-60μm wide, and central rounded or polygonal cells (72-144μm long x 69-120μm wide)(Surget and Barron, 2005) (figure 9) .The starchy endosperm is mainly composed of starch and storage proteins which are needed for growth of the seedling.

Figure 9. Transverse section of outer endosperm (adapted from Fleckinger, 1935).



4.2.2. Major chemical components of wheat grain

Wheat grains are mainly composed of carbohydrates (65-75% starch and fiber) and proteins (7-16%), but also contain lipids (2-6%), water (12-14%) and micronutrients (Pomeranz, 1988). The different compounds are unevenly distributed among grain tissues. Indeed, the starchy endosperm is mainly composed of starch and storage proteins, whereas the germ and outer layers contain most of the fiber, lipids, minerals and bioactive compounds. According to Fardet (2010), whole grain wheat contains about 13 % dietary fiber and at least 2 % bioactive compounds other than fiber (Table 5), which accounts for at least 15 % of the whole grain. In the bran and germ fractions, still higher proportions are reached: about 45 and 18% of dietary fiber, and about 7% and at least 6 % of bioactive compounds respectively; which represents about 52 % and at least 24 % of these fractions.

Table 5. Average content of the major bioactive compounds in whole-grain wheat and wheat bran and germ fractions (%)*. From Fardet (2010).

Bioactive compound	Whole-grain wheat┼	Wheat bran┼	Wheat germ┼
α-Linolenic acid (18 : 3n-3)	╪	0.16	0.53
Sulfur compounds	0.5	0.7	1.2
Total free glutathione§	0.007	0.038	0.270
Dietary fibre¥	13.2	44.6	17.7
Lignins	1.9	5.6	1.5
Oligosaccharides¶	1.9	3.7	10.1
Phytic acid	0.9	4.2	1.8
Minerals and trace elements	1.12	3.39	2.51
Vitamins	0.0138	0.0398	0.0394
B vitamins	0.0091	0.0303	0.0123
Vitamin E (tocopherols and tocotrienols)	0.0047	0.0095	0.0271
Carotenoids	0.0034	0.00072	╪
Polyphenols	0.15	1.1	>0.37
Phenolics acids	0.11	1.07	>0.07
Flavonoids	0.037	0.028	0.300
Lignans	0.0004	0.005	0.0005
Alkylresorcinol	0.07	0.27	╪
Betaine	0.16	0.87	0.85
Total choline	0.12	0.17	0.24
Total free inositols (*myo*-and total *chiro*-inositols)	0.022	0.025	>0.011
Phytosterols	0.08	0.16	0.43
Policosanol + melatonin + *para*-aminobenzoic acid	0.00341	0.00290	>0.00186
Total	>15.4	51.5	>23.9
Subtotal (without dietary fibre)	>2.2	6.9	>6.2

*Mean percentages of bioactive compounds found in wheat bran, whole-grain wheat and wheat germ

┼ Expressed as g/100g food.

╪ No data found.

§ Total free glutathione is given as glutathione equivalents = reduced gluthatione + (oxidized gluthatione x2).

¥ Dietary fibre content is measure according to the AOAC method as such or modified.

¶ Oligosaccharides include fructans, raffinose and stachiose.

4.2.2.1. Macronutrients

Starch is the most abundant cereal polysaccharide and is a major food reserve providing a bulk nutrient and energy source in the human diet. It has some unique properties, which determine its functionality in many food applications, in particular in breadmaking. Starch is composed of two types of polysaccharide molecules amylose and amylopectin. These starch macromolecules exist in granules. Each starch granule contains concentric growth rings of alternating semi crystalline and amorphous composition (Jenkins et al., 1994). In mature wheat, grains starch is deposited in two distinct types of granules: A-type granules (diameter > 9.9µm) and B-type granules (diameter < 9.9µm) (Soulake and morisson, 1985). Differences in the granule size distribution of the two starch granule types in mature wheat grains will find different applications in industrial food and nonfood applications (Dai and al., 2009). Carbohydrates can be classified into two broad categories: available and unavailable (Dewettincl et al., 2008). Available carbohydrates can be digested and absorbed by humans whereas unavailable carbohydrates (dietary fiber) are not hydrolyzed by endogenous human enzymes, although they may be fermented in the large intestine to varying extents. Dietary fiber includes resistant starch (RS), cellulose and other complex polysaccharides, such as arabinoxylans, β-glucans, pectin and arabinogalactans, together with lignin. It promotes beneficial physiological effects including laxation and/or blood cholesterol attenuation and/or blood glucose attenuation (Report of the dietary Fiber Definition Comitee to the board of directors of the American association of cereal chemist, 2001, Vol 46, NO 3). The bread-making potential of wheat is largely derived from the quantity and quality of its protein content. From a functional point of view, two groups of wheat proteins should be distinguished. The *non-gluten proteins* (between 15 and 20% of total wheat protein) are mainly present in the outer layers of the wheat kernel and have a higher level of lysine than the proteins of endosperm. The *gluten-proteins* are found in the endosperm of the mature wheat grain (between 80 and 85% of total wheat protein) where they form a continuous matrix around the starch granules. Gluten-proteins are the major storage proteins of wheat and are distinguished as monomeric gliadins and polymeric glutenins. Gliadins and glutenins have high contents of proline and glutamine, but low contents of lysine and threonine (Goesaert et al., 2005).

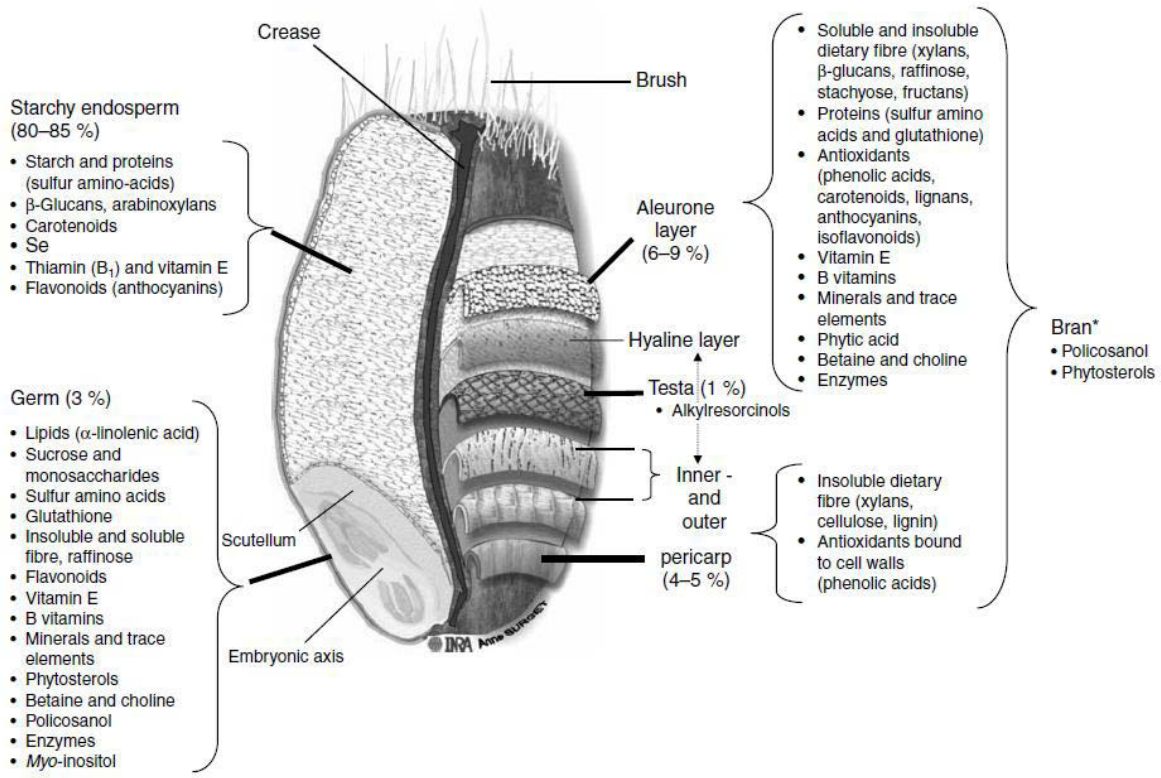
4.2.2.2. Micronutrients

Bioactive compounds such as micronutrients and phytochemicals are also unequally distributed within the grain (figure 10). Indeed, the testa contains almost all of the grain alkyresocinols (Landberg et al, 2007), a class of phenolic lipids reported to exhibit antioxidant properties and anticancer activity (Kozubek and Tyman, 1999). According to Pomeranz (1988), the germ contains B vitamins and exhibits the highest concentration of α-tocopherol, which has been shown to be a powerful antioxidant. Plant sterols, are also concentrated in germ (Nystrom et al, 2007). The aleurone layer represent about 7 % of the wheat

grain mass but contains the major part of the B vitamins and about half the total mineral content (Antoine et al., 2002). Phytate is a naturally occurring compound formed during maturation of plant seed and grains and therefore a common constituent of plant derived food (Abdoulaye and al., 2011). Phytate accumulates in the aleurone layer in wheat grain and is also known for its ability to chelate minerals (especially zinc, calcium, iron and as with protein residue), thus limiting their intestinal bioavailability (Lopez et al., 2002). However this compound has also been found to display strong antioxidant activity *in vitro* (Graf and Eaton., 1990).

Adom et al (2005) reported the phytochemical profiles and antioxidant activity in the endosperm and bran/germ of three different wheat varieties. It appears from their results that in whole wheat flour the bran/germ fraction contributed to 83 % of the total phenolic content, 79 % of the total flavonoid content, 78 % of the total zeaxanthin and 51 % of the total lutein. Some authors have also shown that the aleurone layer is richer in antioxidant compounds than the other bran tissues, mainly due to its high content of phenolic acids (Esposito et al., 2005).

Figure 10. The three wheat fractions (bran, germ and endosperm) with their main bioactive compounds. From Fardet (2010).



Among the micronutrients, vitamins are the second major group of molecules required for the growth and the healthy maintenance of the human body. The action of each of these vitamins is complex and multi-factorial.

4.3. Vitamins

4.3.1. Definition and classification of vitamins

The first scientist who discovered a health disease relative to the lack of a "vitamin" was Dr. William Fletcher in 1905. Doctor Fletcher was looking for the causes of the disease called BeriBeri when he discovered that eating unpolished rice prevented against this disease. William Fletcher believed that the husk of the rice was containing special nutrients. In 1906, English biochemist Sir Frederick Gowland Hopkins also discovered that certain food factors were important for health. It is only from 1912, that a Polish scientist Cashmir Funk named this special nutrient as a "vitamine" after "vita" meaning life and "amine" from functional group contained in the thiamin, he had isolated from rice husks. Vitamine was later shortened to vitamin. Together, Hopkins and Funk formulated the vitamin hypothesis of deficiency disease.

Vitamins are a group of organic compounds which are needed in small quantities for the normal functioning of the human body. They have to be supplied through the diet because they are not synthesized by the human organism. Thirteen vitamins are universally recognized at present, and they are classified into two groups according to their solubility. The fat-soluble vitamins are represented by vitamins A (only some carotenoids can be converted into vitamin A), D, E and K. The water-soluble vitamins which are not stored by the body, comprise vitamin C and the members of the B group, namely thiamin (vitamin B1), riboflavin (vitamin B2), niacin (vitamin B3), vitamin B6, pantothenic acid (vitamin B5), folate (vitamin B9), biotine and vitamin B12. Most of the vitamins exist as group of chemically related compounds having similar biological activity capable of meeting a nutritional requirement (frequently called "vitamers") (Table 6). During this literature review, the vitamin B9 and B8 will not be investigated.

Table 6. Content of major vitamer and carotenoid forms in whole-grain wheat, with brief commentary regarding bioactivity and bioavailability considerations.

Compounds	Chemical forms	Bioactivity and Bioavailability	Content in whole grain (mg/ 100g)
Vitamin E	α-Tocopherol	70 in human as free compounds	1.32
	β-Tocopherol	-	0.68
	δ-Tocopherol	-	-
	ϒ-Tocopherol	-	-
	α-Tocotrienol	-	0.54
	β- Tocotrienol	-	2.69
	δ- Tocotrienol	-	-
	ϒ- Tocotrienol	-	-
Carotenoids	β-carotene (pro-Vit A)		0.005-0.025
	Lutein		0.026-0.383
	Zeaxanthin		0.009-0.0039

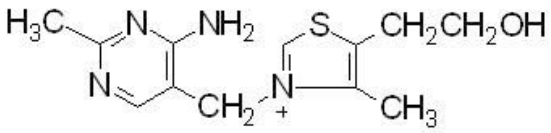
Compounds	Chemical forms	Bioactivity and Bioavailability	Content in whole grain (mg/ 100g)
Thiamin B1	Thiamin	91 in rats/ free thiamin mononitrate	0.13-0.99
	Thiamin phosphates		
Riboflavin B2	Riboflavin	Riboflavin, FAD and FMN have approximately equivalent activity and bioavailability. 95 as oral supplement in human subjects	0.04-0.31
	Flavin mononucleotide (FMN)		
	Flavin adenine dinucleotide (FAD)		
Niacin B3	Nicotinic acid	Low since mostly bound	1.9-11.1
	Nicotinamide		-
	Nicotinamide adenine dinucleotide (NAD)	Highly available	-
	Nicotinamide adenine dinucleotide phosphate (NADP)	Highly available	-
	Nicotinic acid adenine dinucleotide (NAAD)		-
	Nicotinic acid adenine dinucleotide phosphate (NAADP)		-
	Nicotinamide riboside		-
	Nicotinic acid ribosyl		
Pantothenic acid B5	Pantothenic acid	About 50 in human/average American diet	0.7-2
	Coenzyme A (CoA)	Approximately full bioavailability	-
	Phosphopantetheine	Approximately full bioavailability	-
	Pantothenol	Approximately full bioavailability	-
Vitamin B6	Pyrdoxine	71-79 in human/average American diet as compared with free compound	0.09-0.66
	Pyridoxal & Pyridoxamine		-
	$B_6$5'-phoshate vitamers	Similar to bioavailability to nonphosphorylated vitamers	
	Pyridoxine-5'-β-D-glucoside	Approximately 50% (human) bioavailability	0.06-0.29
Biotin B12	Biotin	Very low	0.002-0.011
	Biocytin		
Folate B9	Folate	Often incomplete bioavailability probably due to to food matrix	0.014-0.087
	Dihydrofolates		-
	Tetrahydrofolates (THF)		

4.3.2. Thiamin (Vitamin B1)

4.3.2.1. Chemical properties and biochemical functions

Thiamin is a colorless organosulfur compound with a chemical formula $C_{12}H_{17}N_4OS$. Its structure comprises substituted pyrimidine and thiazole moieties linked by a methylene bridge (figure 11). It is a quaternary amine, which exists as a monovalent or divalent cation depending on the pH of the solution. Three phosphorylated forms of thiamin occur in nature. In living tissues the predominant form is the diphosphate, usually referred to as thiamin pyrophosphate, which serve as a coenzyme in several metabolic pathways. Thiamin triphosphate has no coenzyme function, but it has a role (no completely understood) in nerve transmission (Ball, 2004). Thiamin monophosphate appears to be biologically inactive. Thiamin is the most labile of the various water-soluble vitamins when the pH of the matrix approaches neutrality. Maximum stability in solution is between pH 2.0 and 4.0. Stability of thiamin is also dependent upon the extent of heating and on the food matrix characteristics. Dwivedi and Arnold (1973) found that thermal degradation was primarily due to scission of the methylene bridge yielding pyrimidine and thiazole.

Figure 11. Chemical structure of thiamin



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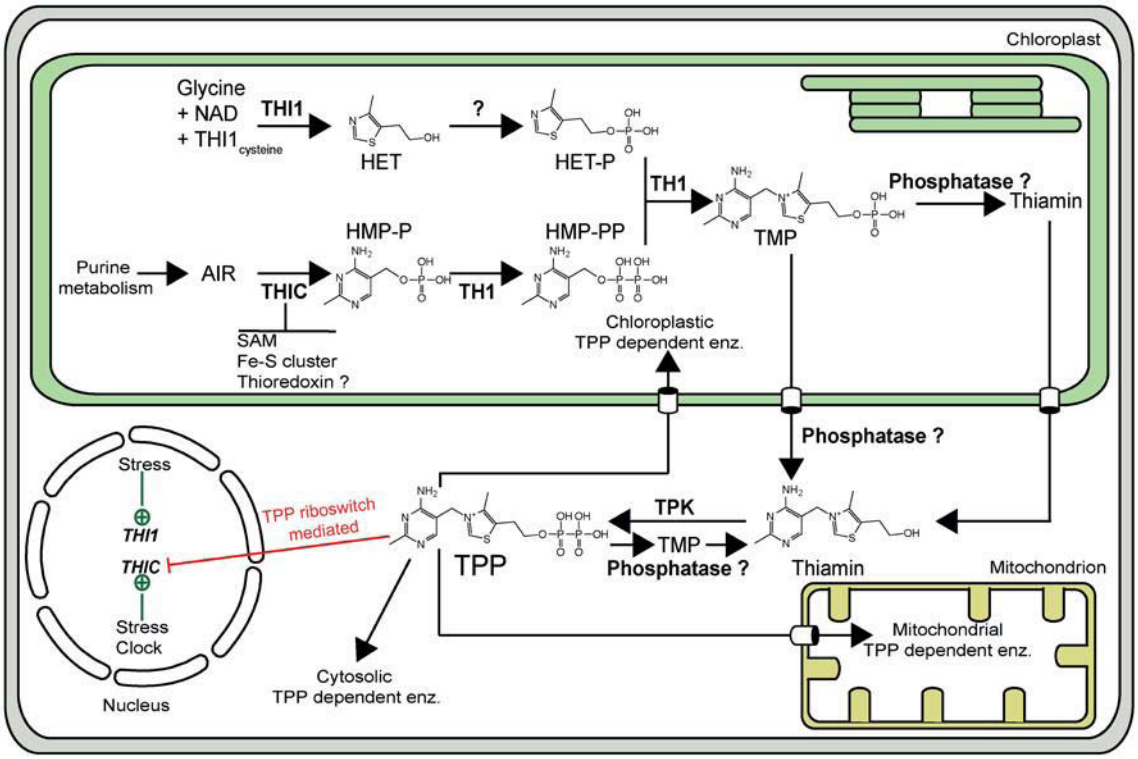
4.3.2.2. Biosynthesis

Synthesis of thiamin in plants involves the independent synthesis of two substituted thiazole and pyrimidine compounds, 4-methyl-5-(2-hydroxyethyl) thiazole phosphate (HET-P) and 4-amino-5-hydroxymethyl-2-methylpyrimidine diphosphate (HMP-PP) which are coupled to form thiamin monophosphate (TMP). The HET-P biosynthesis in plant involves HET-P synthase (THI1) which catalyzes the formation of thiazole moiety from nicotinamide adenine dinucleotide (NAD), glycine and sulfur from a backbone cysteine. The pyrimidine moiety is biosynthesized by a complex chemical rearrangement of 5-aminoimidazole ribonucleotide (AIR) to HMP-P through the action of THIC protein (EC 4.1.99.17) (Raschke et al., 2007). HMP-P is then phosphorylated by a bifunctional protein characterized in maize as THI3 and in *Arabidopsis* as TH1 (EC 2.7.4.7; EC 2.5.1.3) (Rapala-Kozik et al., 2007). The same protein catalyzes the condensation step between HET-P and HMP-PP to produce TMP. All of these steps have been shown to occur in the chloroplast (Raschke et al., 2007). As TMP is not directly phosphorylated to thiamin diphosphate (TPP), it is assumed to be subsequently dephosphorylated to thiamin by broad specificity

phosphatases (Pourcel et al., 2013). Indeed, a broad substrate acid phosphatase has been isolated from maize and biochemically characterized showing it can dephosphorylate TMP. However, the same phosphatase showed relatively higher specificity toward TPP (Rapala-Kozik et al, 2009). Thus, it has been suggested that TPP can also be dephosphorylated to thiamin. On the other hand, thiamin can be pyrophosphorylated to make the active cofactor TPP by thiamin pyrophosphokinase (Rapala-Kozik et al., 2009). Thiamin diphosphate thus formed binds to metabolite binding domain within certain messenger RNA's called riboswitches. This leads to allosteric rearrangement of the messenger RNA structures that results in modulation of gene expression and protein production (figure 12).

Figure 12. The thiamin biosynthesis pathway in *Arabidopsis thaliana* (from Pourcel et al., 2013)



4.3.2.3. Dietary sources and bioavailability

Dietary sources

All plant and animal tissues contain thiamin, and so the vitamin is present in all natural unprocessed food. Rich source of vitamin thiamin include yeast and yeast extract, wheat bran, oatmeal, whole-grain cereals, pulses, nuts, lean pork, heart, kidney and liver (Ball, 2004). Thiamin is stored in the non phosphorylated form and even in mature seeds the phosphate esters represent only 5% of the total thiamin content. The long-term thiamin storage in wheat seeds is the one

bound to proteins and represent 45% of the total thiamin content (Ndaw et al., 2000). Whole-grain wheat is particularly significant sources of thiamin compared with other food sources. Furthermore, wheat germ is classified as high sources of thiamin (Bock, 2000).

Bioavailability

Few studies have been conducted on the bioavailability of thiamin present naturally in foods, but it is generally considered to be high (Ball, 2004).

4.3.2.4. Syndromes caused by Thiamin deficiency

The combined results from several human studies have shown that inducement of thiamin deficiency in adults produces a wide range of disorders involving the gastrointestinal tract, central and peripheral nervous systems, and cardiovascular system (Ball, 2004). Well known syndromes caused by thiamin deficiency include beriberi, Wernicke-Korsackoff syndrome and optic neuropathy.

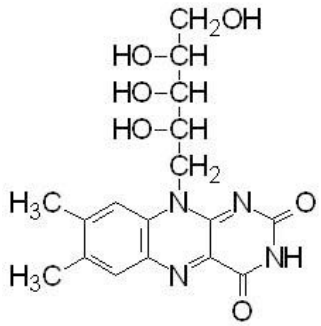
4.3.3. Riboflavin (Vitamin B2)

4.3.3.1. Chemical properties and biochemical functions

The name riboflavin comes from ribose (the sugar which forms part of its structure) and flavin, the ring-moiety which imparts the yellow color to the oxidized molecule (from Latin *flavus*, "yellow"). Riboflavin is heat-stable but alkali-labile and photo-labile. It is stable towards acids and oxidizing agents. Riboflavins are commonly used as additives by the food and canning industry, in particular to fortify infant foods, fruit juices and milk.

Riboflavin belongs to the class of flavins (isoalloxazines) which are among the most widely investigated class of compounds in view of their biological functions, nutritional importance, therapeutic uses and photosensitivity. Chemically riboflavin is 3, 10-dihydro-7, 8-dimethyl-10-[(2S, 3S, 4R)-2, 3, 4, 5-tetrahydroxypentyl] benzopteridine-2, 4-dione (figure 13). The biologically active forms of riboflavin are its coenzyme derivatives, namely flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). FMN and FAD serve as coenzymes in a wide variety of reactions in intermediary metabolism. Flavoproteins catalyze dehydrogenation reactions as well hydroxylations, oxidative decarboxylations, dioxygenations, and reductions of oxygen to hydrogen peroxide. Thus, many different kinds of oxidative and reductive reactions are catalyzed by flavoproteins (conversion of tryptophan to niacin, production of pyridoxic acid from pyridoxal or reduction of the oxidized form of glutathione (GSSG) to its reduced form (GSH)). In addition, riboflavin nutrition may be critical in regulating the rate of inactivation of lipid peroxides (Rivlin, 2007).

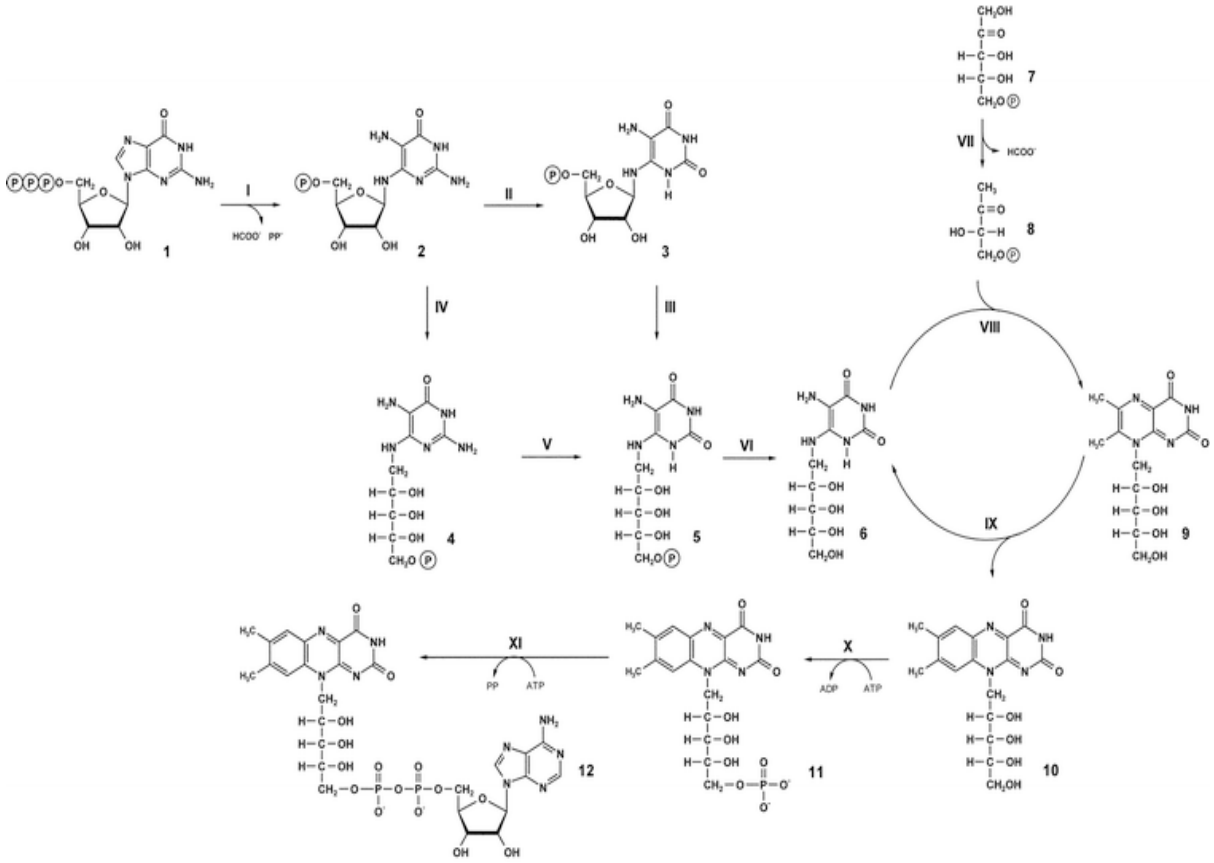
Figure 13. Chemical structure of Riboflavin



4.3.3.2. Biosynthesis

The biosynthesis of one molecule of riboflavin (figure 14) requires one molecule of guanosine-5'-triphosphate (GTP) and two molecules of ribulose phosphate (Fischer and Bacher, 2006). No cofactors are required except divalent metal ions and nicotinamide adenine dinucleotide phosphate (NADPH). The different reactions are catalyzed by GTP cyclohydrolase II (EC 3.5.4.25); pyrimidine deaminase (EC 3.5.4.26); 3-4-dihydroxy-2-butanone-4-phosphate synthase (EC 4.1.99.12); lumazine synthase (EC 2.1.5.78) and riboflavin synthase (EC 2.5.1.9).

Figure 14. Biosynthesis of riboflavin and flavoconenzymes in plants. (From Fischer and Bacher, 2006)



Step **I**, GTP cyclohydrolase II; step **II**, 2,5-diamino-6-ribosylamino-4 (3H)-pyrimidinone 5'-phosphate deaminase; step **III**, 5-amino-6-ribosylamino-2,4 (1H, 3H)-pyrimidinedione 5'-phospate reductase; step **IV**, 2,5-diamino-6-ribosylamino-4 (3H)-pyrimidinone 5'-phosphate reductase; step **V**, 2,5-diamino-6-ribitylamino-4 (3H)-pyrimidinedione 5'-phosphate deaminase; step **VI**, hypothetical phosphatase; step **VII**, 3,4-dihydroxy-2-butanone 4-phosphate synthase; step **VIII**, 6,7-dimethyl-8-ribityllumazine synthase; step **IX**, riboflavin synthase; step **X**, riboflavin kinase; step **XI**, FAD synthetase; **1**,GTP; **2**, 2,5-diamino-6-ribosylamino-4 (3H)-pyrimidinone 5'-phosphate; **3**, 5-amino-6-ribosylamino-2,4 (1H, 3H)-pyrimidinedione 5'-phospate; **4**, 2,5-diamino-6-ribosylamino-4 (3H)-pyrimidinone 5'-phosphate; **5**, 2,5-diamino-6-ribitylamino-4 (3H)-pyrimidinedione 5'-phosphate; **6**, 5-amino-6-ribitylamino-2,4 (1H,3H)-pyrimidinedione; **7**,ribulose-5-phosphate; **8**, 3-4-dihydroxy-2-butanone-4-phosphate; **9**, 6,7-dimethyl-8-ribityllumazine; **10**, riboflavin; **11**, FMN; **12**, FAD

4.3.3.3. Dietary sources and bioavailability

Dietary sources

Riboflavin is widely distributed as an essential constituent of all living cells. The most relevant dietary sources are milk and milk products, meat, eggs, fish and green leafy vegetables (Souci et al., 2008).
Natural grain products tend to be relatively low in riboflavin, but fortification and enrichment of grains and cereals have led to a considerable increase in riboflavin intake from these food items (Rivlin, 2007).

Several factors in food preparation and processing may influence the amount of riboflavin that is actually bioavailable from dietary sources. In view of the light sensitivity of riboflavin, it is not surprising that appreciable amounts of riboflavin may be lost with exposure to UV light, particularly during cooking and processing (Batifoulier et al., 2005). However, whole wheat bread could provide 20% of the daily requirement of riboflavin (Rivlin and Pinto, 2001).

Bioavailability

Most of the riboflavin in dietary sources occurs largely in the form of their coenzymes derivatives (FAD/FMN); these molecules must be hydrolyzed before absorption. They are actually released as free riboflavin by digestive enzymes in the small intestine and absorbed in the bloodstream. The bioavailability of riboflavin is high, probably about 95% (Zempleni et al., 1996) but our capacity to absorb riboflavin from the small intestine is only moderate.

4.3.3.4. Syndromes caused by Riboflavin deficiency

In humans, signs and symptoms of riboflavin deficiency (ariboflavinosis) include cracked and red lips, inflammation of the lining of mouth and tongue, mouth ulcers, crack at the corners of the mouth (angular cheilitis), and a sore throat. A deficiency may also cause dry and scaling skins, fluid in the mucous membranes and iron-deficiency anemia. Riboflavin deficiency is classically associated with the oral-ocular-genital syndrome.

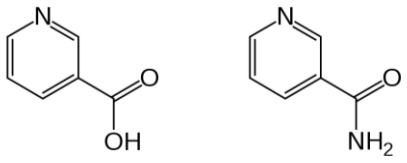
4.3.4. Niacin (Vitamin B3)

4.3.4.1. Chemical properties and biochemical functions

Niacin is referred to as vitamin B3 because it was the third of the B vitamins to be discovered. It has historically been referred to ”vitamin PP” or “PP-factor”, that are derived from the term “pellagra preventive factor”. Interestingly, niacin is derived from **ni**cotonic **a**cid and vita**min**.
The term niacin is often defined as nicotinic acid (pyridine-3-carboxylic acid) (Figure 15), although it can be defined more expansively as “nicotinamide (pyridine-3-carboxamide) (Figure 15), nicotinic acid and derivatives that exhibit the biological activity of nicotinamide” (standing committee, 1998). During this literature review, “Niacin” will be used in reference to all forms with vitamin activity, while “nicotinic acid” will refer to pyridine-3-carboxylic acid.

Nicotinic acid cannot be directly converted to nicotinamide, but both compounds are precursors of the coenzymes nicotinamide adenine dinucleotide (NAD+)and nicotinamide adenine dinucleotide phosphate (NADP+) *in vivo* (Cox et al., 2000). It is the C-4 position on the pyridine ring of the nicotinamide moiety that participates in oxidation and reduction reactions. Due to the electronegativity of the amide group and the nitrogen at position 1 on this ring, hydride ions can readily reduced the oxidized position C-4. This is the basis for the enzymatic hydrogen transfer reactions that are ubiquitous among organisms (Kirkland and Rawling, 2001). NAD+ and NADP+ are the biologically active form and are coenzymes for many dehydrogenases, participating in many hydrogen transfer process. Such reactions are not destructive in the sense that NAD^+ and NADH are interconverted by hydride transfer. In the other hand, NAD^+ is also a substrate for three classes of enzymes that cleaves NAD^+ to produce nicotinamide and an ADP-ribosyl product. As depicted in (figure 16), the three classes of NAD^+ consumers are: (i) ADP-ribose transferases or poly(ADP-ribose) polymerase, (ii) cADP-ribose synthases and (iii) sirtuins (type III protein lysine deacetylases) (Belenky et al, 2007). With respect to the non-redox functions of NAD+, the glycosidic linkage between nicotinamide and ribose is a high-energy bond, and cleavage of this bond drives all types of ADP-ribose transfer reactions in the forward direction. As mentioned by Noctor et al. (2011), understanding and manipulation of the NAD+ biosynthesis may offer interesting perspectives to enhance the nutritional value of plants.

Figure 15. Chemical structures of niacin compounds



Nicotinic acid (Na) Nicotinamide (Nam)

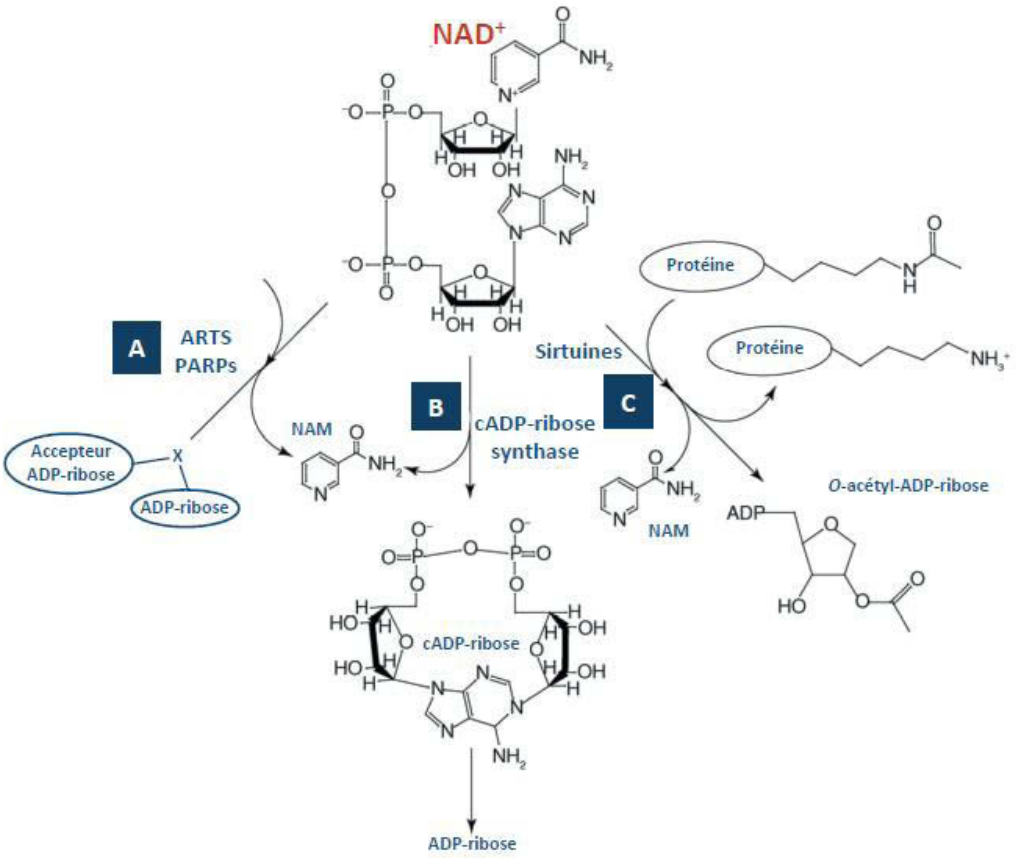
4.3.4.2. Biosynthesis

Among the biosynthetic routes known to exist in nature for nicotinic acid biosynthesis, higher plants use the dihydroxyacteone phosphate/aspartate pathways which are the so-called *de novo* pathway. The first isolatable intermediate is quinolic acid (QA). This 2, 3-pyridine-dicarboxylic acid is then metabolized into nicotinic acid mononucleotide (NaMN) by a quinolinate phosphoribosyltransferase (QPT, EC 2.4.2.19). NAD is synthesized from NaMN by a nicotinate mononucleotide adenyltransferase (NaMNAT, EC 2.7.7.18) and a NAD synthetase (NADS, EC 6.3.5.1) (Ashihara et al., 2005).

Nicotinamide (NAM) is formed as a catabolite of nicotinamide adenine dinucleotide (NAD) and is a key metabolite of pyridine metabolism (Matsui et al., 2007) (figure 16).

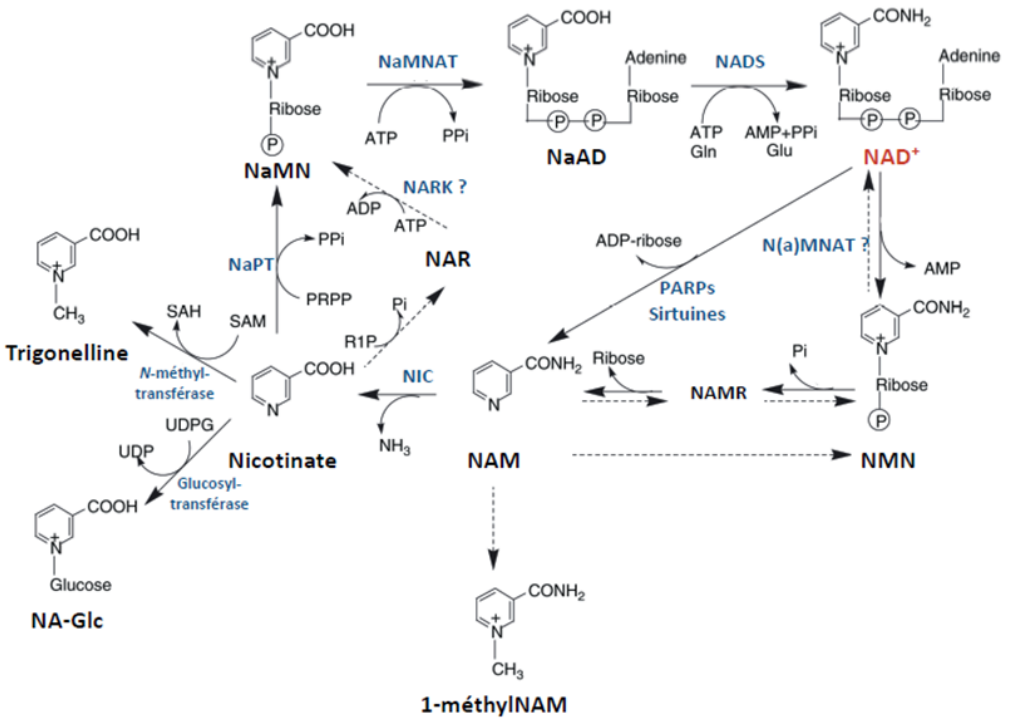
Besides synthesizing NAD^+ *de novo* from simple amino acid and precursors, cells can also use the salvage pathway which aims to synthesize NAD^+ (figure 17) from nicotinic acid, nicotinamide or nicotinamide riboside. In plant, nicotinamide is first deaminated by a nicotinamidase (NIC, EC 3.5.1.19) to nicotinic acid (which is not the case in mammals and explained the exogenously supplied of nicotinic acid as diet) and salvaged by nicotinate phosphoribosyl transferase (NaPT, EC 2.4.2.11) to nicotinate mononucleotide (NaMN) (Revollo et al., 2004).
Metabolism pathway of nicotinamide mononucleotide (NMN) is still little known and no plant genes are known for NMN nucleosidases, which hydrolyses NMN to nicotinamide (figure 17) (Gerdes et al., 2012).
In addition to salvage, nicotinamide and nicotinic acid are used for the synthesis of alkaloids such as nicotine and ricinine in very limited plants such as tobacco and castor bean plant for instance. (Katoh and hashimoto, 2004). A simple and ubiquitous alkaloid is derived from nicotinic acid in the one step-conversion to trigonelline by an N-methyl transferase (EC 2.1.1.7). Matsui et al. (2007) surveyed the concentration of trigonelline in seeds of various plant species but did not find any accumulation of this compound in wheat seeds. In some plant species, nicotinic acid1 N-glucoside is formed (Upmeier et al, 1988c). Mason and Kodicek (1973) demonstrated that a partial hydrolysis product of the bound nicotinic acid of wheat bran was nicotinoyl glucose. In mature cereal grains most of the niacin is present as bound nicotinic acid and is concentrated in the aleurone layer and germ layers (Ball, 2004).

Figure 16. The NAD+-consuming enzymes pathway (from Belencky et al., 2007)



NAD+ as a substrate for ADP-ribose transfer, cADP-ribose synthesis and protein lysine deacetylation. **(A)** ADP-ribose transferases (ARTs) and polyADP-ribose polymerases (PARPS) transfer ADP-ribose from NAD^+ as a protein modification with production of nicotinamide. In the case of PARPs, the ADP-ribose acceptor, X, can also be ADP-ribose, forming poly(ADP-ribose). **(B)** cADP-ribose synthases cyclize the ADP-ribose moiety of NAD+ with production of NAM. These enzymes also hydrolyze cADP-ribose. **(C)** Sirtuins use the ADP-ribose moiety of NAD+ to accept the acetyl modification of a protein lysine, forming deacetylated protein plus NAM and, after acetyl group rearrangement, a mixture of 2' and 3' O-acetylated ADP-ribose. Each NAD+ consuming enzyme is inhibited by the NAM product.

Figure 17. The Nicotinamide salvage pathway (From Matsui et al., 2007)



Dotted arrows in the schematic diagram indicate possible reactions and thus further experimental is needed. Gln: glutamine; Glu: glutamate; UDP: uridine diphosphate; NA-Glc: nicotinate glucoside; NAR: nicotinate riboside; NARK: NAR kinase; UDPG: UDP glucose; R1P: riboside-1- phosphate; SAH: S-adenosyl-methionine

4.3.4.3. Dietary sources and bioavailability

Dietary sources

Niacin can be synthesized in the human body from the essential amino acid L-tryptophan, but most of the daily requirement must be met by external by external sources of niacin. Approximately 60mg of L-tryptophan yield 1mg of niacin. Because of the contribution of tryptophan, foods containing balanced protein are important contributors to total niacin intake. Lean red meat, poultry and liver contain high levels of both niacin and tryptophan and, together with vegetables are important sources of the vitamin (Ball, 2004). Whole-grain wheat and especially its bran and germ fractions are particularly significant sources of nicotinic acid (Fardet, 2010). Ndaw et al. (2002) reported the absence of nicotinamide from wheat germ. In relatively unprocessed food, particularly

meats, NAD^+ and $NADP^+$ are considered to provide the largest part of the available niacin (Lahély et al., 1999).

Bioavailability

The majority of the bound nicotinic acid in mature cereals grains is biologically unavailable after conventional cooking (Wall and Carpenter, 1988). Mason and Kodicek (1973) suggested that the incorporation of Nicotinoyl glucose within indigestible celluloses and hemicelluloses prevents access of the gastrointestinal esterases to the nicotinoyl ester bonds. Alternatively, esterase activity may be poor: the methyl ester of nicotinic acid was only 15% effective as the free acid in supporting the rat's growth (Wall and Carpenter, 1988). In addition it has never been possible to release the nicotinic acid bound forms in wheat flour or in wheat germ, even partially, by subjecting these foodstuff samples, to a mild treatment (pH2, 37°C, 3 h), close to the physiological conditions of digestion (Ndaw et al, 2002). It was shown that hot alkali treatment of corn (Kodicek et al., 1956) or wheat bran (Wall and Carpenter, 1988) before baking was enough to release the nicotinic acid from its bound forms. An important additional finding is the changes in the distribution of niacin compounds in corn during its development. Immature sweet corn is an effective source of available niacin (due to the predominance of NAD+ and NADP+), whereas niacin in mature field corn is largely unavailable (due to bound forms of niacin) (Wall and Carpenter, 1988).

4.3.4.4. Syndromes caused by niacin deficiency

Niacin deficiency symptoms are exhibited only by humans and dogs among the mammals. The disease caused by deficiencies of the vitamin niacin is "pellagra" in man and "canine black tongue" in dogs. Pellagra is characterized by diarrhea, dermatitis, dementia as well as "Casal's necklace" lesions on the lower neck, inflammation of the mouth and tongue, amnesia, delirium and eventually death if left untreated (Wall and Carpenter, 1988).

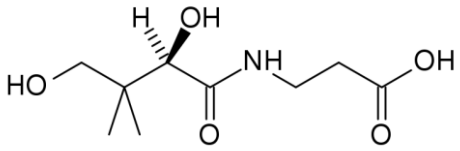
4.3.5. Pantothenic acid (Vitamin B5)

4.3.5.1. Chemical properties and biochemical functions

Pantothenic acid ($C_9H_{17}NO_5$) also called "yeast factor", is an amide of pantoic acid and β-alanine (figure 18), and was first isolated by Roger J. Williams in 1938 from yeast and liver concentrates. This compound is stable to oxidizing and reducing agents but is degraded by heating in an acid and alkaline medium. Pantothenic acid is vital to the synthesis and maintenance of coenzyme A (CoA), a cofactor and acyl group carrier for many enzymes processes, and acyl carrier protein (ACP), a component of the fatty acid synthase complex. CoA is important in energy metabolism for pyruvate to enter the tricarboxylic acid cycle (TCA cycle) as acetyl CoA, and for α-ketoglutarate to be transformed to succinyl-CoA in the cycle (Gropper et al., 2009).

Pantothenic acid in the form of CoA is also required for acylation and acetylation, which for example are involved in signal transduction and enzyme activation and deactivation, respectively (Gropper et al., 2009)

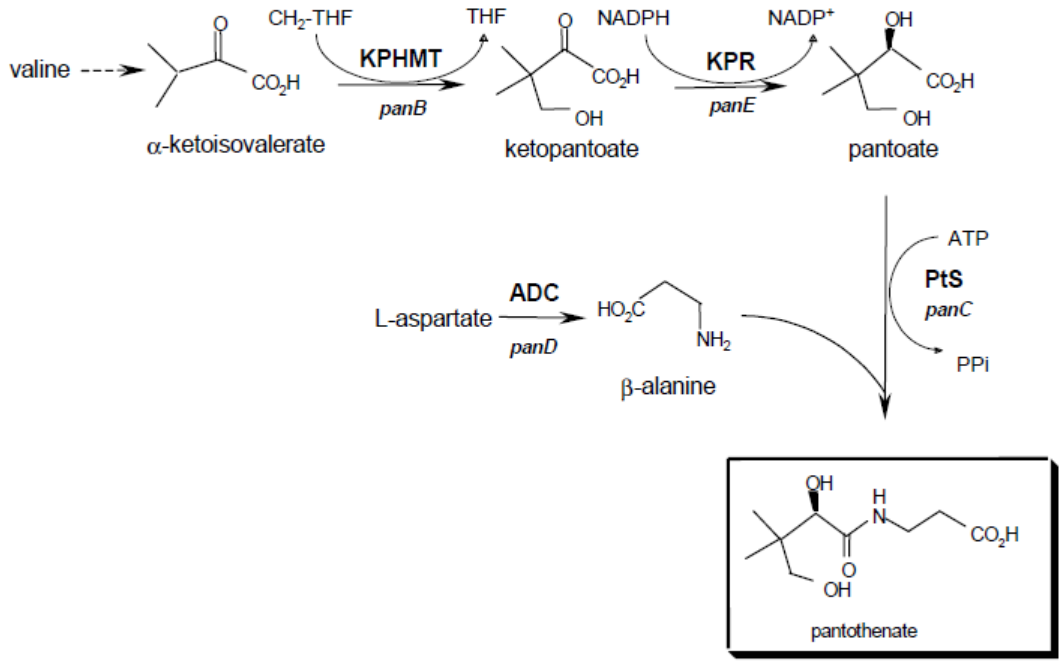
Figure 18. Chemical structure of Pantothenic acid.



4.3.5.2. Biosynthesis

The understanding of the biosynthetic pathway of pantothenic acid in plants is still incomplete. This is in contrast to the situation in bacteria such as *Escherichia coli,* in which the pathway is well established (Figure 19). In *E. coli*, the first committed step is the formation of ketopantoate from the oxoacid of valine, by the enzyme ketopantoate hydroxymethyltransferase (KPHMT). Ketopantoate is then reduced to pantoate by ketopantoate reductase (KPR). In a separate branch β-alanine is synthesized from L-aspartate by the enzyme L-aspartate-α-decarboxylase (ADC).Finally, pantothenate is produced during a condensation reaction between pantoate and β-alanine, catalyzed by pantothenate synthase (PtS) (Ottenhof et al., 2004). Identification, characterization and subcellular targeting of KPHMT and PtS were described from *Arabidopsis thaliana* by Ottenhof et al. (2004). Direct evidence of the presence of KPR in plants was provided by Jones et al. (1994). In addition a putative *Arabidopsis* oxidoreductase with some similarity to KPR was identified (Ottenhof et al., 2004).

Figure 19. Pantothenate biosynthetic pathway in E.coli



The pathway comprises four different enzymes: KPHMT (EC 2.1.2.11); KPR (EC 1.1.1.169); ADC (EC 4.1.1.11) and PtS (EC 6.3.2.1). The genes encoding each enzyme are shown in italic (from Ottenhof et al., 2004).

4.3.5.3. Dietary sources and bioavailability

Dietary sources

The major food source of pantothenic acid is meat. Whole-grain cereals are particularly significant sources of pantothenic acid, and especially its bran and germ fractions.

Bioavailability

Little information is available on the bioavailability of dietary pantothenic acid. Values of 41 to 60 percent (mean 50 percent) have been given for absorbed food-bound pantothenic acid from the “average American diet”.

4.3.5.4. Syndromes caused by pantothenic acid deficiency

Pantothenic acid deficiency is extremely rare and has not been thoroughly studied. Symptoms of deficiency are irritability, fatigue and apathy due to low CoA levels. Some neurological symptoms can also appear as acetylcholine synthesis will be impaired.

4.3.6. Pyridoxine (Vitamin B6)

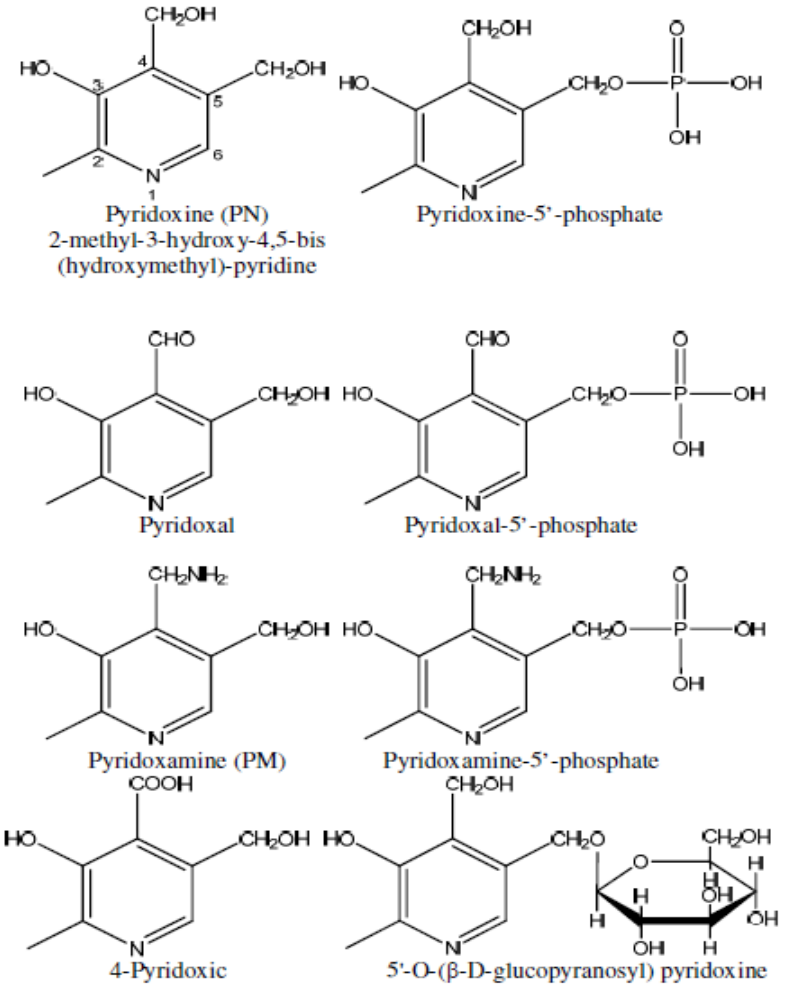
4.3.6.1. Chemical properties and biochemical function

The formula of vitamin B6 was first published by Ohdake in 1932. He worked on the isolation from rice-polishing of what he called "Oryzanin" (Thiamin) and found vitamin B6 as a by-product. In general, all forms of vitamin B6 are quite stable at acidic pH when light is excluded, but exposure to heat and light (especially UV or near UV irradiation) results in significant degradation, the degree of which increase as the pH approaches alkalinity.

Vitamin B6 includes a group of 3-hydroxy-2-methyl-pyridine derivatives that can exist in the C-4' position as an alcohol (Pyridoxine) (Pn), as an aldehyde (Pyridoxal)(PL) and an amine (Pyridoxamine)(PM)(figure 20). While all the three vitamers form can exist in free, phosphorylated, or bound to proteins, it is pyridoxal 5'-phosphate (PLP) that is the biologically most active form which is used as a cofactor for many important enzymatic reactions. These PLP-dependant enzymes catalyze more than 140 distinct enzymatic reactions (Mooney et al., 2009).

Many of the PLP-dependant enzymes catalyze important steps in the amino acid metabolism, like transamination, racemization, decarboxylation and α, β-elimination reactions. PLP represents also an important cofactor for the degradation of storage carbohydrates, such as glycogen. Besides these roles as a cofactor, vitamin B6 may play a crucial role in protecting cells from oxidative stress because the vitamin has been shown to exhibit antioxidant activity that even exceeds that of vitamins C and E (Bilski et al., 2002).

Figure 20. Chemical structure of pyridoxine and related compounds



4.3.6.2. Biosynthesis

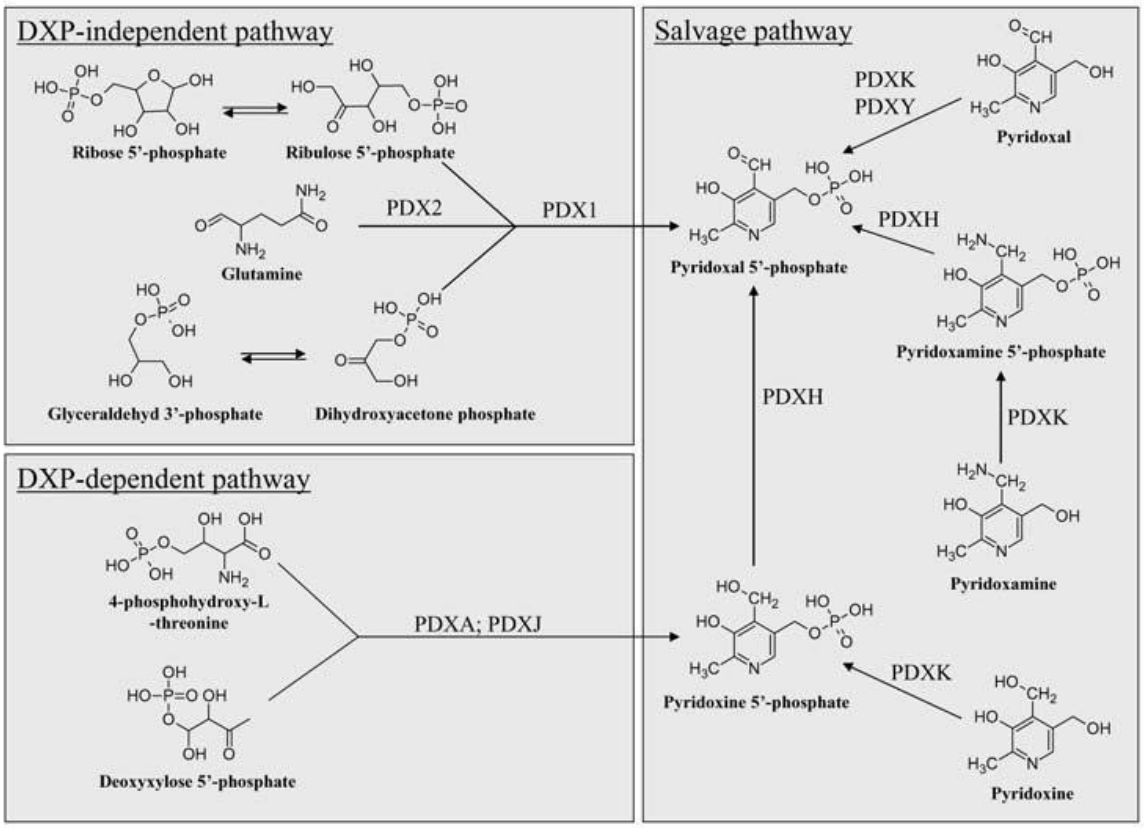
Two existing pathways are known for *de novo* vitamin B6 biosynthesis.The deoxyxylose 5'-phosphate (DXP)-dependant pathway, which is present in eubacteria, such as *Escherica Coli.*

The second one, the DXP-Independant pathway is described for some bacteria, archaea and eukarya. The occurrence of this pathway is demonstrated in plants and involves the two cytosolic pyridoxine biosynthesis proteins (PDX1/PDX2). These two synthase proteins directly synthesize PLP from ribose-5'-phosphate or ribulose-5'-phosphate, in combination with either glyceraldehyde-3'-phosphate or dihydroxyacetone phosphate and glutamine (Figure 21) (Mooney et al, 2009). In addition to the direct synthesis of new PLP, the vitamers are interconvertible via the so called *salvage pathway*. These conversions (pyridoxamine-5'-phosphate (PMP) to PLP or pyridoxine-5'-phosphate (PNP) to PLP) are made in the (cytosol or chloroplast) by the salvage enzyme pyridoxamine-5'-phosphate

oxidase (PDXH). Two different salvage enzymes kinases (Figure 21) can phosphorylate PN, PL and PM to their respective 5'-phosphate (Fitzpatrick et al, 2011; Mooney et al., 2009). The two kinases differ in their substrate specificities, with pyridoxal kinase (PDXY) acting on PL, whereas PDXK can utilize all three non-phosphorylated vitamers as substrate. Most eukaryotes contain a single kinase (PDXK).

Besides the biosynthesis of vitamin B6, catabolism of the vitamin is also an important aspect for cellular homeostasis of the compound (Mooney et al, 2009). PLP, PMP and PNP can be dephosphorylated to pyridoxal, pyridoxamine, and pyridoxine respectively, which can be rephosphorylated by pyridoxal kinase in the cytosols or plastids (Gerdes et al, 2012). Pyridoxal is reduced by a chloroplastic pyridoxal reductase (EC 1.1.1.65) to pyridoxine (Herrero et al, 2011), which can be glucosylated by a UDP-glucose-dependent glucosyltransferase (EC 2.4.1.160). Neither the phosphatase(s) that hydrolyse PLP, PMP and PNP nor the glucosyltransferase(s) that form pyridoxine glucoside have been identified (Gerdes et al, 2012).

Figure 21. Pathways of pyridoxine synthesis and salvage. PDX1/2 (EC 1.4.3.5); PDXK (EC 2.7.1.35) (from Mooney et al., 2009)



4.3.6.3. Dietary sources and bioavailability

Dietary sources

Whole-wheat flour is an important source of vitamin B6 but the presence of glycosylated forms of pyridoxine complicates their nutritional evaluation.

Glycosylated forms of pyridoxine range from 5% to 75% of the total vitamin B6 in fruit, vegetables and grains. In data reported by Sampson et al. (1996), there was a significant fraction (average of 68%) of vitamin B_6 in wheat present as pyridoxine glucoside. Pyridoxine is the primary, possibly the only, glycosylated form of vitamin B_6 (figure 20), with no evidence of glycosylated pyridoxal or pyridoxamine (Gregory, 1998).

Bioavailability

Pyridoxine glucoside exhibits only 50 % bioavailability in humans and it also exerts weak antagonist effects on the utilization of non-glycosylated pyridoxine (Gregory, 1998). The overall bioavailability of vitamin B6 in a mixed diet has been reported approximately 75% (Tarr et al., 1981).

4.3.6.4. Syndromes caused by vitamin B6 deficiency

Vitamin B6 deficiency can contribute to adverse health effects because of the many metabolic roles played by PLP. The classical clinical syndrome for vitamin B6 deficiency is a seborrhoeic dermatis-like eruption, atrophic glossitis with ulceration, conjunctivitis and neurological symptoms of somnolence, confusion and neuropathy. Vitamin B6 deficiency is also associated with insufficient activities of the coenzyme pyridoxal phosphate which result in impaired tryptophan-niacin conversion.

4.3.7. Vitamin A and carotenoids

4.3.7.1. Chemical properties and biochemical function

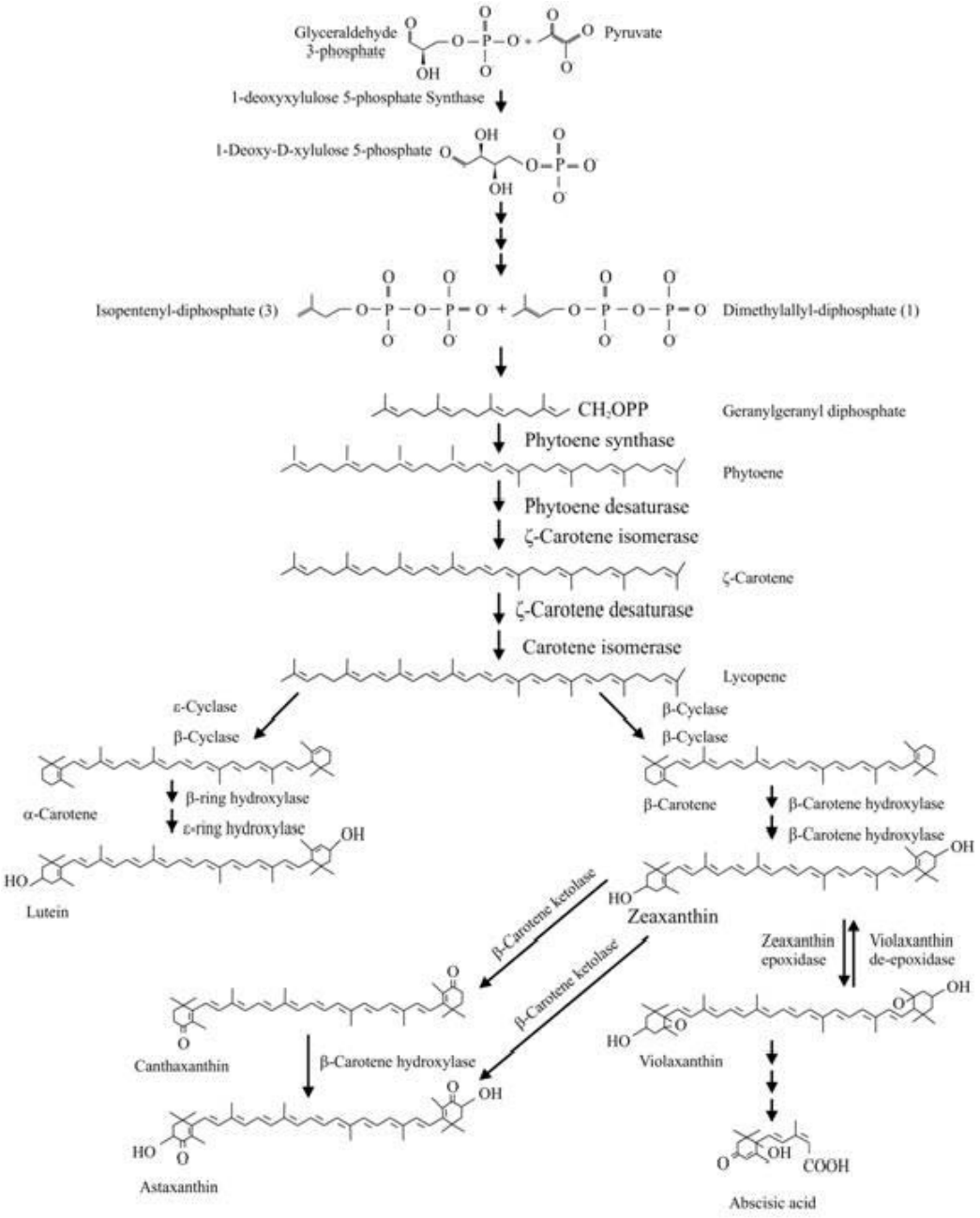
The name vitamin A was first used in 1920 to signify the early discovery of the growth factor and to differentiate it from water-soluble vitamins, collectively called vitamin B at that time. Carotenoids are the most widespread pigments in nature with yellow, orange and red colors, and have also received substantial attention because of their role as provitamins and as antioxidants. The term “provitamin A” is accepted to differentiate carotenoids precursors of vitamin A from carotenoids without vitamin A activity. The predominant carotenoid in wheat, is lutein, followed by zeaxanthin (Leenhardt et al, 2006) (figure 22). These two carotenoids have no vitamin A activity whereas β-carotene and α-carotene are provitamin A carotenoids.

Carotenoids belong to the category of tetraterpenoids as they are being built from four terpene units each containing 10 carbon atoms. Carotenoids are classified into hydrocarbon (carotenes) and their oxygenated derivatives (xanthophylls). The most characteristics feature of carotenoids is the long series of conjugated double bonds forming the central part of the molecule. This gives them their shape, chemical activity, and light-absorbing properties. Carotenoids in plants provide pigmentation essential for photosynthesis, reproduction and protection. They may also act as antioxidants in lipid environments of many biological systems through their ability to scavenge most reactive oxygen species (Liu, 2007)

4.3.7.2. Biosynthesis

Carotenoid biosynthesis begins with the condensation of two molecules of geranylgeranyl pyrophosphate (GGPP or GGDP) to form phytoene, catalyzed by the enzyme phytoene synthase (*Psy*)(EC 2.5.1.32) (figure 22). Phytoene then undergoes four sequential reactions (which requires at least four enzymes) to form lycopene. These enzymes are phytoene desaturase (PDS, EC 1.3.3.5) and zeta-carotene desaturase (ZDS, EC 1.3.3.6), which produce respectively poly-*cis*-compounds, which are then isomerized to trans form by zeta-carotene isomerase (ZISO, EC 5.2.1.12) and carotenoid isomerase (CRTISO, EC 5.2.1.13) to produce lycopene. Lycopene cyclization then occurs, following one of two pathways that incorporate either the ε-ring (EC 5.5.1.18) or the β-ring (EC 5.5.1.19) in two step reactions. The asymmetrical carotenes formed after the first set of reactions undergo further cyclization to form ε-carotene, α-carotene or β-carotene (Cunningham and Gantt, 1998). Carotenoid hydroxylase enzymes (EC 1.14.13.129) specific to the ε-ring and β-ring catalyze the double hydroxylation of α-carotene and β-carotene to form lutein and zeaxanthin respectively. Zeaxanthin can undergo reversible double epoxidation of the rings, mediated by the enzyme zeaxanthin epoxidase (ZEP, EC 1.10.99.3), to form violaxanthin, a precursor to abscisic acid.

Figure 22. Carotenoid biosynthesis pathways (From Hannoufa and Hossain, 2011).



4.3.7.3. Dietary sources and bioavailability

Dietary sources

Fruits and vegetables provide most of the carotenoids in the human diet. Wheat is a useful source of antioxidant compounds. In bread wheat, however, the concentration of carotenoids is low (from 0.1 to 2.4 μg/g dm) but they are more abundant in durum wheat (1.5 to 4.0 μg/g dm) (Panfilli et al, 2004). Generally,

lutein is the carotenoid present in the highest concentration in wheat, followed by zeaxanthin and then β-cryptoxanthin. Thus, wheat is not a sources of vitamin A.

Bioavailability

Many factors such as age, gender, genetic factors, health status, the food matrix, and the amount and types of carotenoids in the meal affect the bioavailability or efficiency with which provitamin A carotenoids are absorbed in the intestine (Ortiz-Monasterio et al, 2007). However, the bioavailability of provitamin A carotenoids in specific foods is not well understood.

4.3.7.4. Syndromes caused by vitamin A deficiency

Vitamin A deficiency continues to be a widespread public health problem despite some success in supplementation/fortification programs, and has been linked to adverse health outcomes including night blindness, corneal scarring and blindness (Ortiz-Monasterio et al., 2007). With relations to dentistry, a deficiency in vitamin A leads to enamel hypoplasia.

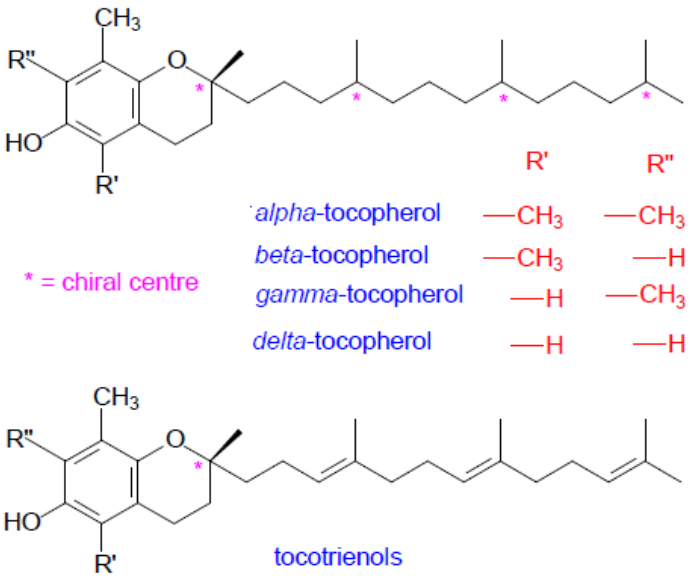
4.3.8. Vitamin E

4.3.8.1. Chemical properties and biochemical function

Vitamin E is a generic term that constitutes a group of structurally related compounds comprising of two vitamers i.e. tocopherol and tocotrienol. Tocols occurs in eight forms (α-tocopherol, β-tocopherol, γ-tocopherol, δ-tocopherol) and (α-tocotrienols, β-tocotrienols, γ-tocotrienols, δ-tocotrienols) as shown in figure 23. Their basic structures comprise a 6-hydroxychroman group and a phytyl side chain made of isoprenoid units. The chroman group may be methylated at different positions to generate different compounds with vitamin activity. Tocopherol and tocotrienol have similar structures except that tocopherols contain saturated phytol side chains while tocotrienols have three carbon-carbon double bonds in the phytol side chain.

The most important chemical property of tocols is their antioxidant activity. They are primary antioxidants that act by donating hydrogen atoms to lipid peroxyl radicals, thus retarding propagation of lipid oxidation. Tocols are also effective quenchers for singlet oxygen and nitrogen oxide radicals (Lampi et al., 2008)

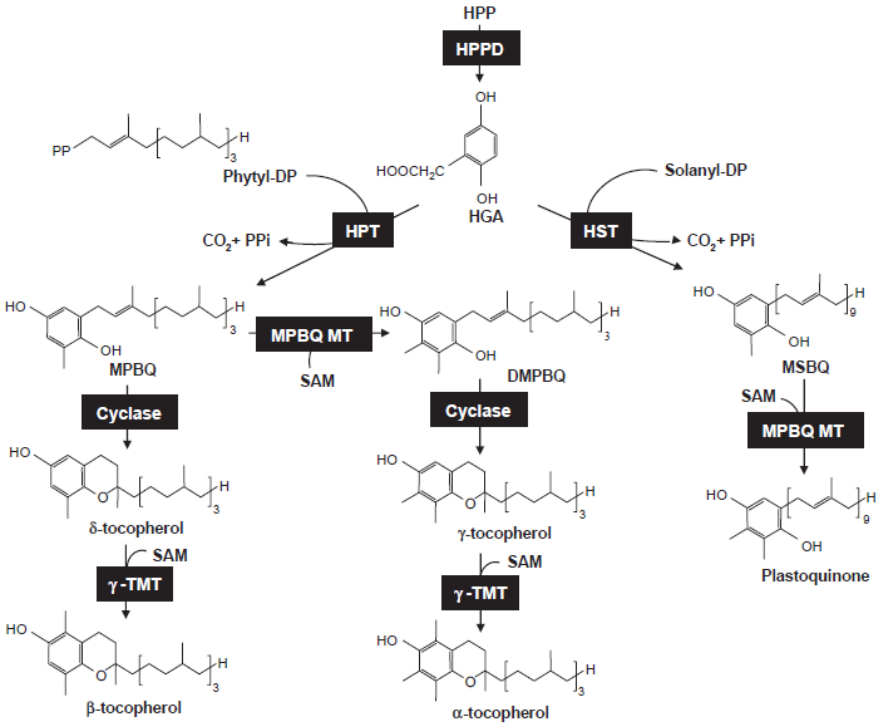
Figure 23. Chemical structures of Tocopherols and Tocotrienols



4.3.8.2. Biosynthesis

The plant tocopherol pathway utilizes cytosolic aromatic amino acid metabolism for synthesis of the chromanol group (homogentisic acid-HGA) and the plastidic deoxyxylulose 5-phospate pathway for synthesis of the side chain (phytyl-diphosphate phytil DP for tocopherols and geranyl-geranyl diphosphate GDDP for tocotrienols) (figure 24). The first step in tocopherol synthesis involves the production of the aromatic head group, HGA from p-hydroxyphenylpyruvic acid (HPP) by the enzyme p-hydroxyphenylpyruvic acid dioxygenase (HPPD, EC 1.13.11.27). HGA is then subject to prenylation with phytyl-diphosphate or geranyl-geranyl diphosphate to yield 2-methyl-6-phytylplastoquinol (MPBQ) and 2-methyl-6-geranylgeranylplastoquinol (MGGBQ), respectively, by the enzyme homogentisate prenyltransferase (HPT, EC 2.5.1.115; EC 2.5.1.116). MPBQ and MGGBQ are the first committed intermediates in the synthesis of all tocopherols and tocotrienols, respectively. The substrate specificity of HPT is a key factor determining whether tocopherols, tocotrienols or both compounds can be made in an organism. The next steps in synthesis are ring methylations (EC 2.1.195) and/or ring cyclization (EC 5.5.1.24) (Dellapenna, 2005).

Figure 24. Tocopherol biosynthesis in plants (from Dellapenna, 2005).



4.3.8.3. Dietary sources and bioavailability

Dietary sources

Tocols are found in many foods including vegetable oils, nuts, grains, fruits and vegetables. Among the various sources of tocols cereal grains are considered to be only moderate sources. Generally in bread wheat grains, α- and β-tocols are the major vitamers present, and there are more tocotrienols than tocopherols (Lampi et al., 2008).

Bioavailability

Very little is known about the bioavailability of tocols from grains and grain product. It has been suggested that it may be difficult to release tocols from grains and seeds, because they are poorly digested, whereas the bioavailability from oils and fats is considered to be much better (Stahl et al., 2002). However, bioaccessibility of α-tocopherol from wheat bread is very high, being 99.6 ± 11.3% and that from wheat germ almost as good, being 53.29 ± 7.85% (Reboul et al., 2006).

4.3.8.4. Syndromes caused by vitamin E deficiency

Vitamin E deficiency is rare and is almost never caused by a poor diet. However vitamin E deficiency causes nerve problem due to poor conduction of electrical impulses along nerves due to changes in nerve membrane structure and function.

5. Approach to improve wheat vitamin content

5.1. Influence of growing conditions, genetics and processing on nutritive value

Whole wheat grain provide important micronutrients such as minerals, vitamins, fibers, phytochemicals but also are a rich source of proteins, lipids and energy thanks to their high starch content, making them nutritionally benefic for human consumption. However, it has been suggested that intensive wheat breeding has resulted in decreased health benefits, by focusing on yield and processing quality. As an example, Fan et al. (2008) have reported an important decrease in the content of essentials minerals, including iron and zinc in modern high-yielding cultivars compared to older varieties. Nevertheless, even if the decline in the contents of bioactive compounds could be corrected by genetic approach or growing condition, the matter of how those grains need to be technologically process to satisfy the nutrition of the consumers is an important issue which has to be considered. Indeed, processing may decrease or increase the levels of the macronutrients and micronutrients in wheat grains and also modify their bioavailabilty.

As reported by Shewry et al. (2010), the high heritability of several major groups of bioactive components (arabinoxylan fiber, tocols and sterols) means that these are realistic targets for plant breeders to produce novel cultivars with enhanced health benefits. However, the amount of the most highly heritable components is affected by environmental conditions. This poses a challenge to food processors (in addition to the study of the impact of industrial processing on bioactive compounds) who wish to provide products with higher nutritional value as it may not only be necessary to source specific varieties but also to routinely determine the contents of bioactive components in specific grain samples. Therefore, the development of simple and economical methods for determining composition is crucial to monitor intake and facilitate product development.

5.1.1. Growing conditions

In a recent study, Shewry et al. (2011) reported that the content of thiamin, niacin and pyridoxine were positively correlated with the mean temperature from heading to harvest, but not with precipitation during grain development. On the contrary, riboflavin was positively correlated with precipitation during the 3 months prior to heading. Other bioactive compounds have shown to be affected by environmental factors. Indeed, Shewry et al. (2010) showed that folates, sterols and tocols were strongly correlated with the mean temperature between heading and harvest. Davis et al. (1984) have shown modifications of thiamin concentration greater than 20 % due to growing location differences. The use of fertilizers can increase the cereal content of Se (Hawkesford and Zhao, 2007) but do not seem to affect riboflavin concentration (Pomeranz et al., 1988). In addition, abiotic or/and biotic stress during wheat seedlings, may be responsible for the generation of low molecular mass antioxidants. This has been shown with α-tocopherols and

carotenoids in wheat seedling under temperature and salt-stressed environments (Keles and öncel, 2012).

5.1.2. Grain selection and genetic approach

Success in crop improvement through plant breeding strategies depends on the existence of genetic variation for the target traits in the gene pool available to the breeder (Ortiz-Monasterio et al., 2007). In general, we found little variation for carotenoid levels (mostly within 0.43-1.74 µg/g DM) (Leenhardt et al, 2006), for bioavailable niacin levels (0.16-1.74µg/g DM), for pyridoxine levels (1.27-2.97µg/g DM) and for riboflavin levels (0.77-1.40µg/g DM) (Shewry et al, 2011) in whole wheat flour. On the other hand, the high heritability of thiamin (30%), reported by (Shewry et al, 2011), indicates that increases in content of this vitamin may be possible by breeding. Furthermore, the evaluation of 175 genotypes of different wheat types grown under similar conditions for tocopherol and tocotrienol contents (Lampi et al, 2008) has shown a large variation. This genetic diversity of tocopherol and tocotrienols in wheat genotype may be exploited by plant breeders to improve the nutritional quality of whole-wheat grains. Because of the low natural variation of B vitamins and carotenoids, transgenic approaches may be the only viable way to achieve nutritionally meaningful gain B vitamin and carotenoid concentrations in wheat. This approach is unlikely to be used for B vitamins, at least in the short term, as their biosynthetic pathways are complex and their genetic control and regulation are not well understood (Webb et al., 2007). The use of a transgenic approach for developing bread-wheat with high carotenoid contents is not envisioned in the short term (Ortiz-Monasterio et al., 2007) because (1) the yellow-orange color of the resulting flour would have significant acceptability problems in most regions where wheat is consumed and (2) most carotenoids would probably be oxidized and lost because of processing during flour production, or baking may finally produce foods with lower than desirable levels of carotenoids.

5.1.3. Influence of processing

5.1.3.1. fractionation processes

Wheat flour milling aims to separate the starchy endosperm from the outer layers and germ with the highest possible yield and purity. In general, these peripheral tissues are removed because they may confer unwanted properties to foods in terms of safety, processing, or acceptability by consumers. This is related to the presence of contaminants in the bran fractions of wheat grains (mycotoxins, pesticides residues, heavy metals, etc.) or to the intrinsic properties of the tissues or of their components (e.g. instability of germ lipids). However, since recent studies have revealed the potential health benefits of bioactive compounds located in the so-called bran fraction, new milling

technologies have been search for producing flours with enhance nutritional values.

Physical and biochemical pretreatments

Before milling or grinding, whole-wheat grains can be subjected to different pretreatments in order to either modify their mechanical properties to improve tissue dissociation and increase or decrease the tissue fragmentation, or to modify their biochemical properties, and thus influence the tissues composition and/or biological activity (Hemery et al., 2007). The different physical pretreatments which can be applied are: changes in the moisture content of grain and bran, modifications in the temperature of conditioning and exposure to UV radiation (Peyron et al., 2002).

In addition to physical pretreatments, biochemical pretreatments, by including chemical agents or enzymes in the tempering water, can also induce changes in the tissue composition and influence the physical properties of grains and bran (Hemery et al., 2007). Different ways to pre-hydrolyse insoluble fiber (for example, insoluble β-glucans or arabinoxylans) into soluble fiber with endohydrolase (Napolitano et al., 2006) or ester bound ferulic acid with feruloyl-esterases (Fauld and Williamson, 1995) have been considered. Moore et al. (2006) tested the influence of enzymatic treatment of wheat bran on the release of insoluble bound antioxidants such as phenolic phytochemicals. The use of such pretreatment aims to liberate as much as 50% of the insoluble bound ferulic acid, and also shows that such methods may help to improve the bioaccessibility of phenolic compounds in wheat bran. Chemical agents can also be used during biochemical pretreatments of whole-wheat grains. Desvignes et al. (2006) studied the effect of soaking wheat grains in calcium chloride solutions on the mechanical properties of the outer layers. This grain pretreatment enhance the endogenous peroxidase activities, and thus induced a modification of the phenolic profile of the cell walls. These changes suggest that modification of the mechanical properties occurs at the interface between the aleurone layer and the surrounding tissues.

Natural or traditional pretreatments

In order to improve tissue dissociation and enhance the tissue fragmentation, the use of traditional and natural processes such as germination, soaking and/or fermentation in a highly hydrated medium has been examined. Before fermentation, whole-grain cereals are generally soaked, germinated, dried and coarsely ground with a grinding stone (Gadaga et al., 1999). Wall and Carpenter (1988), have reported that the alkaline soaking of whole corn used by the Mexican population have rendered grain niacin more nutritionally available.

Lastly, fermentation of whole-grain cereals have been reported in several studies to increase the content of available methionine and B vitamins, such as thiamin, riboflavin, niacin, folates and pantothenic acid through the action of micro-organisms (Chavan and Kadam, 1989).

The pre-processing of whole wheat grains allows separating and removing the embryo, the scutellum, a large part of the outer pericarp and the other peripheral layers prior to milling in order to improve the efficiency of the milling step and enhance the quality of the flour.

Pre-processing of wheat grains: Degerming

Germ as a rich source of tocopherols and other micronutrients might be a valuable ingredients to fortify food products. During traditional roller milling, only 15-20 % of the 3% of germ in wheat grain is recovered. Posner and Li, (1991a and b), showed that removing the germ before milling allows recovery of almost the entire germ. This pre-processing, also prevents the oxidative damage of flour (enhancing storage stability) produced when milling the grain with germ, as germ is known to contain an important amount of lipids and oxidative enzymes. Degerming prior to milling is more complicated in the case of wheat processing than in the case of corn processing, because of the smaller size of wheat germ. Posner and Li, (1991a and b), have developed a method which allowed the recovery of 50-100% of both the embryonic axis and scutellum, depending on the cultivar and type of wheat.

Pre-processing of wheat grains: Debranning

Debranning is a treatment usually used on rice but has been adapted for wheat (Figure 25b). The debranning of wheat is a pre-milling treatment that allows a controlled and progressive removal of the grain's layers. Debranning of grains can be carried out by friction (peeling), by abrasion (pearling), or by a combination of these two operations (Hemery et al., 2007). The friction mainly removed the peripheral layers of wheat kernels while the abrasion removed the bran tissues. During debranning the conditioning time is reduced to around 20 minutes compared to conventional (Figure 25a) where the grains are kept in water for 12-36 hours. An important issue which has to be considered when milling wheat grain is the contamination by microorganisms and heavy metals which are mainly present in the more peripheral layers of the grains. The use of debranning technique aims to dramatically reduce the number of bacteria and moulds present in wheat grains (Laca et al, 2006). However, as a tempering step is included before debranning in most of the processes, the by-products of debranning may not be easily used as ingredients, because the isolated bran layers are hydrated and require to be dried in order to be stored. In addition the composition of the fractions obtained by debranning shows that they are blends of different histological tissues, and that the main limitation of getting distinct

bran fractions by these processes results from the ovoid shape of wheat grain (Hemery et al., 2007).

Figure 25 a. The conventional durum wheat milling approach (adapted from Ranieri, 2011).

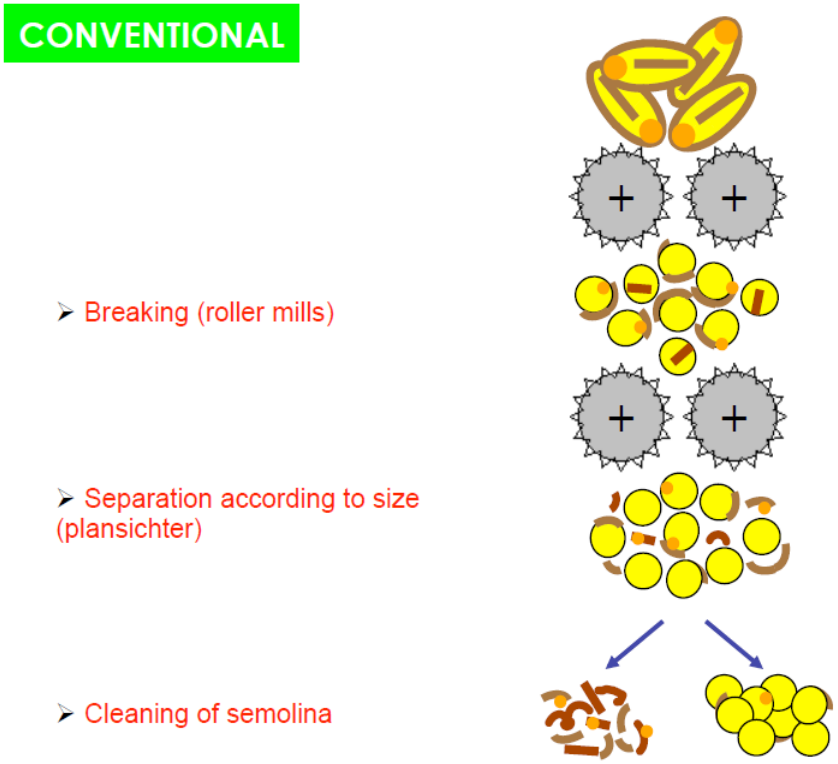
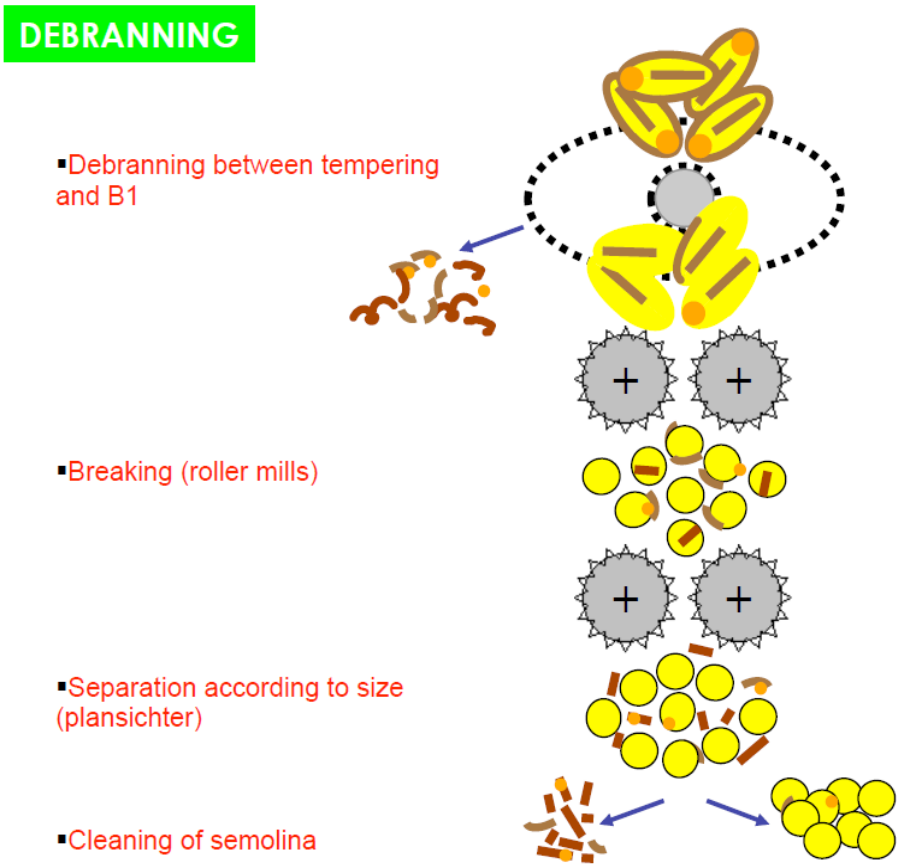


Figure 25 b. The Debranning pre-processing approach of durum wheat grains (From Ranieri, 2011).



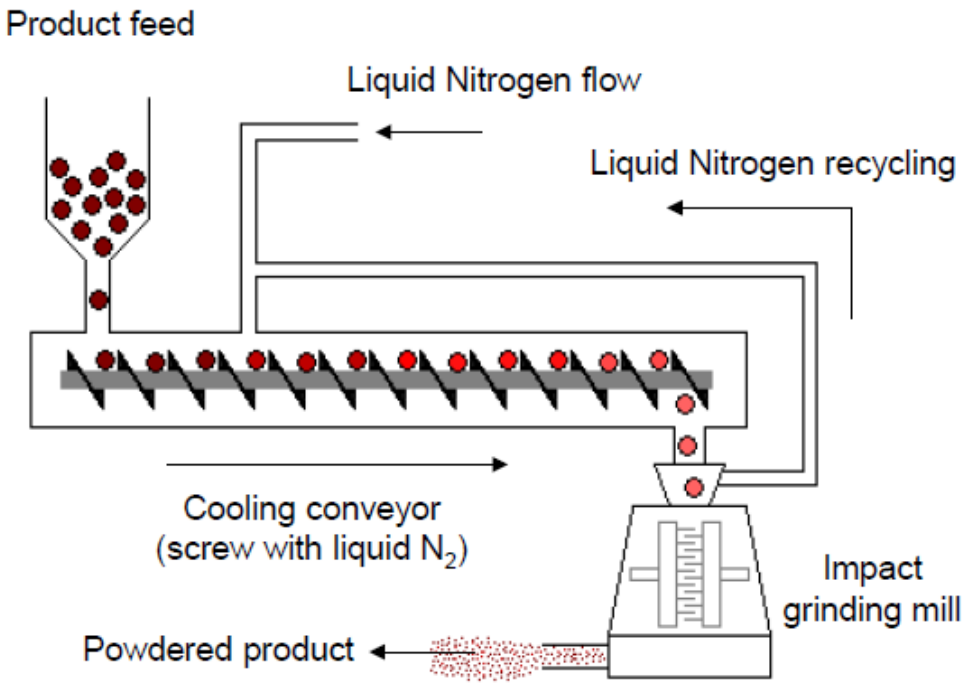
The debranning becomes the first step, before the B1 grinding. The almost part of the bran and of the germ is removed in advance and only a residual amount – the bran located in the kernel's crease - will be separated during the following milling steps.

Bran fractionation

New fractionation approaches allow the separation of the interesting parts of the bran from the undesirable ones and make bioactive compounds more accessible (Hemery et al., 2007). The first approach called 'histological fractionation' aims to dissociate the different constitutive layers of bran to get fractions rich in only one particular tissue of interest (generally the aleurone layer) (Hemery et al., 2007). According to publications from Bühler A.G. (Laux et al., 2006), this process is able to produce an aleurone product which contains 95 wt % of intact single-cell aleurone layer. The second approach called 'macromolecular fractionation' aims to fractionate the different bran tissues and isolates their subcellular constituents (Hemery et

al., 2007). Different methods such pin-milling and jet-milling allow the cell bran tissues to be grind in superfine particule (< 50µm) (figure 26). Another way to obtain very fine particles is to combine cryogeny with impact grinding to increase the brittleness of the materials (Hemery et al., 2007). Nevertheless this new approach of macromolecular fractionation still needs to be improved as the effect of such fine grinding on the nutritional and technological properties of aleurone and other tissues are not well understood. Indeed some studies (Cheng et al., 2006) have shown that micronized bran was more sensitive to heat stress when compared to bran and that the reduction of the particle size may accelerate the loss of natural antioxidants in wheat bran during storage.

Figure 26. Superfine milling installations (From Hemery et al., 2007).



Combinations of impact grinding (here by pin-milling) with a preliminary cryogenic pretreatment of grains in a screw conveyor.

5.1.3.2. Breadmaking

Wheat (*Triticum aestivum*) is by far the most important crop for breadmaking because of its higher baking performance in comparison with all other cereals. A lot of scientific studies have been conduced to evaluate the impact of processing for manufacturing attractive wheat grain product. Most available literature reveals that the levels of micronutrients such as B and E vitamins in a finished product depend upon the food processing operations carried out.The

breadmaking process consists of three major stages: mixing, fermentation and baking.

The mixing and kneading step

Few studies have reported the impact of mixing and kneading on vitamin content. Tabekhia and D'Appolonia (1978) showed that an increase in thiamin, riboflavin, and niacin occurred after mixing the flour into a dough with the remaining bread ingredients. The increase in riboflavin level was also demonstrated by Batifoulier et al. (2005) as upon yeast addition, dough was immediately exhibited a much higher content of riboflavin, showing the strong contribution of yeast riboflavin. On the contrary, kneading have a detrimental impact on pyridoxine and thiamin content (Batifoulier et al., 2005). Leenhardt et al. (2006) noticed that the carotenoids content of bread wheat decreased by about 66% after the kneading step and assumed this loss depends mainly on lipoxygenase activity. Moreover, a reduction in kneading time and intensity associated with a longer period of dough fermentation may spare carotenoids and vitamin E by limiting oxygen incorporation.

The fermentation step

The fermentation process generally increases the B group vitamin concentration in fermented/functional foods. Pederson et al. (1989) showed that the fermentation step slightly increased the contents of some B vitamins. Batifoulier et al. (2005) also found that the thiamin content was increased when fermentation time was prolonged and that the increase was significantly higher in white bread with yeast than with sourdough despite comparable vitamin contributions by the microorganisms. The sequential measurement of pyridoxine during breadmaking shows that long fermentation with yeast led to a significant increase in pyridoxine (Batifoulier et al., 2005). The same authors reported also that whole bread made with yeast results in 30% enrichment in riboflavin, provided that the fermentation is sufficiently long, while sourdough fermentation did not lead to any enrichment of riboflavin. Yeast is also able to compensate for folate losses in wheat bread not only by its high intrinsic folate content but also by synthesizing folates (Kariluoto et al., 2004). As proposed by Leblanc et al. (2013) it is conceivable that the food industry will exploit novel and efficient microbial strains to produce fermented product with enhance nutritional value.

The baking step

Processing and cooking conditions cause variable losses of vitamins. Losses vary widely according to cooking method and type of food. Vitamin degradation depends on specific parameters during the culinary process, e.g., temperature, oxygen, light, moisture, pH, and obviously length of exposure. It is well known that pyridoxal and pyridoxamine are easily destroyed by oxygen and heat, whereas the pyridoxine form is more stable (Leklem, 2001). According to Perera

et al. (1979) the three forms in white bread are present in equal amounts even though, in whole meal bread, the predominant form is pyridoxine. Thiamin is highly unstable at alkaline pH. Stability depends on the extent of heating and on the food properties. Ball (1994) reported that alkaline pH during cooking or processing leads to extensive thiamin losses. Riboflavin is stable to heat and atmospheric oxygen, especially in an acid medium, so losses reported in the literature after a heating process correspond to only about 10% (Batifoulier et al., 2005). Along the B group vitamins, niacin is the most stable one. Results from Tabekhia and D'Appolonia (1978) confirm the high stability of this vitamin to heat and indicate that niacin is retained regardless of the baking procedure. Carotenoids are well known to rapidly lose their activity when heated in the presence of oxygen, especially at higher temperature (Leskova et al., 2006). The baking stage resulted in carotenoids losses of about 36 % (Leenhardt et al., 2006). The same authors studied the evolution of wheat tocols content during breadmaking and reported that baking led to an approximately 15 % loss of tocopherols without significant differences between the three different wheat species studied.

The efficiency of wheat selection, grinding processes or industrial processing might be a key to enhance the nutritional value of wheat-food products. However, to this aim, the development of high throughput methods to determine the composition of vitamins within the different wheat fractions or end-uses products has been explored.

5.2. Analytical methods for the determination of water-soluble and fat-soluble vitamins and carotenoids

Measurement of vitamins in foods is difficult and represents a complex analytical problem for several reasons. Firstly, the diverse chemical structures and properties of vitamins make it very difficult to develop a single method for their simultaneous determination. Secondly, vitamins occur in wheat at relatively low level (Fardet, 2010) which means that the analytical methods have to be very sensitive to determine their contents. Finally the susceptibility of vitamins to degradation by exposure to light, air, heat, alkaline pH and their diverse occurring forms render their extraction from food matrices very challenging.

5.2.1. The water-soluble vitamins

5.2.1.1. *Extraction methods*

The extraction and clean-up represent the rate-limiting stage in almost all vitamin analyses. Each water-soluble vitamin requires specific conditions for its extraction from food. The choice of these conditions depends on the stability of each vitamin (i.e. on its sensitivity to pH, temperature, exposure to light, oxygen partial pressure) and on its bond to the food matrix (Gentilli et al., 2008). Among the diverse methods suggested, vitamins are most often determined in the free form, involving hydrolysis of the phosphorylated form and those bound to proteins and polysaccharides. The extraction of thiamin and riboflavin usually

consists of an acid hydrolysis and an enzymatic treatment. The hydroloric acid treatment (0.1 M HCl in a water bath a 100°C or in an autoclave at 120°C) permits to release the vitamins from their association with the proteins and, secondarily from starch as a strong acidic condition convert this polysaccharide into soluble sugars (Ndaw et al., 2000). This step is usually followed by an enzymatic treatment which allows the dephosphorylation of the vitamins. For economic reasons, the enzymes selected are almost always diastases possessing a phosphatase activity (Ndaw et al., 2000). In a similar way, hot hydrolysis of the sample by a dilute mineral acid (Ball, 1994, 1998) has been used to determine the supposedly bioavailable niacin (nicotinc acid, nicotinamide, nicotinamide adenine dinucleotide (NAD+) and nicotinamide adenine dinucleotide phosphate ($NADP^+$). The main justification for the treatment in acid medium (0.1M) on a water bath at 100°C or in an autoclave at 120°C was the conversion of NAD+ and NADP+ into nicotinamide. However, such a treatment is far removed from the physiological conditions of digestion and would moreover be likely to release, at least partially, the nicotinic acid not bioavailable contained in wheat (Hepburn, 1971). Ndaw et al. (2002) have proposed the replacement of the usual acid treatments by specific extraction of niacin using an enzyme such as NAD glycohydrolase. The extraction protocols suggested for the determination of vitamin B6 in the free form are more varied. Some authors (Bognar, 1985) have simply recommended dephosphorylation by means of sulfuric acid or of an acid phosphatase (Bognar and Ollilainen, 1997). Other authors have suggested the use of a takadiastase combined with a β-glucosidase in order to determine the glycosylated form of vitamin B6 (Bognar and Ollilainen, 1997). In some cases, the enzymatic treatments were preceded by mineral acid hydrolysis (Bognar and Ollilainen, 1997), but Ndaw et al. (2000) have demonstrated the uselessness of an acid treatment before enzymatic hydrolysis for the determination of vitamin B1, B2 and B6. Pantothenic acid may be found in foodstuffs in free and bound forms (coenzyme A (CoA) and acyl carrier protein (ACP)). Its release from the bound forms cannot be performed by chemical hydrolysis, since the amidic bond of pantothenic acid is sensitive to low and high pH (Gentilli et al., 2008). The only practicable alternative is enzymatic digestion (alkaline phosphatase and pantetheinase) which releases pantothenic acid from CoA but not from ACP (Gonthier et al, 1998).

5.2.1.2. *Methods of analysis*

Microbiological assays

The official methods for the determination of water-soluble vitamins are based on procedures (mainly microbiological assays) established for more than 25 years (Blake, 2007). All the B-group vitamins can be assayed microbiologically. Microbiological assay uses the growth response of various vitamin-dependant *Lactobacilly*. For many years, these techniques have been regarded as the “gold standard” since they offer a biological response to vitamin activity (Blake, 2007). In general the precision and accuracy of the various microbiological assays is not high; a relative measurement uncertainty of ± 20% is fairly common. Often,

vitamins laboratories experience a significant percentage of failed analytical runs owing to poor growth or contamination.

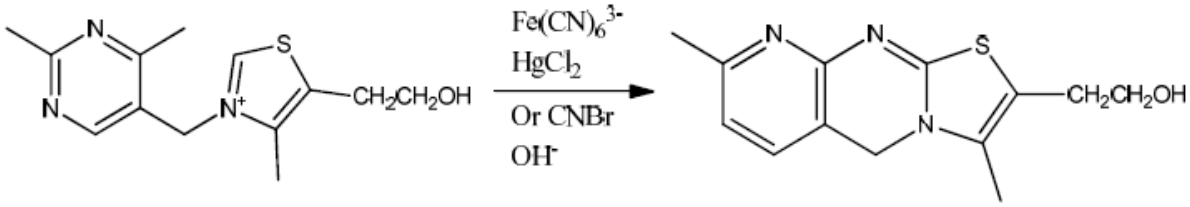
Biosensor/ELISA

Biospecific methods have also been developed such as immunoassays and protein-binding assays. Immunoassays include radioimmunoassay and ELISA, while protein-binding assays utilize naturally occurring vitamin-binding proteins with either radiolabels or enzyme labels. Several reviews have shown the usefulness of these techniques. (Blake, 2007; Kalman et al., 2006)

Spectroscopic methods

The quantification of water-soluble vitamins in fortified foods and supplements has resulted in a large number of methods. At present, the most common methods used are fluorimetry and spectrophotometry. Fluorimetry is particularly useful in pharmaceutical analysis, and the lack of fluorescence of many compounds has led to the development of reagents which aid the formation of fluorescent derivatives. The thiochrome method used for thiamin determination involves its oxidation in alkaline solution and extraction of the thiochrome formed which is measured fluorimetrically (figure 27). Riboflavin is usually assayed by fluorimetrically by the measurement of the characteristic yellowish green fluorescence. The pyridine ring of the nicotinic acid can be opened using cyanogen bromide, and the fission product is coupled with sulphanilic acid to yield a yellow dye at 470 nm (Ötles, 2008). Fluorimetric methods utilizing the fluorescence capacity of vitamin B6 compounds are more sensitive and selective than the spectrophotometric one. Pyridoxal, pyridoxine, pyridoxamine and their phosphorylated counterparts pyridoxine phosphate and pyridoxamine phosphate exhibit significant fluorescence while only pyridoxal phosphate is weakly fluorescent (Ubbink, 2000).

Figure 27. Thiochrome reaction



Chromatographic procedures

Among different analytical techniques, high pressure liquid chromatography (HPLC) is a current popular method which was first utilized for the analysis of vitamins in 1970s. Briefly, this analytical technique involves the injection of a small volume of liquid sample into a tube packed (column) with tiny particles in diameter called the stationary phase. The compounds of the sample are forced to move down the column with a liquid (mobile phase) by high pressure

delivered by a pump. These compounds are separated from one another by the column packing that involves various chemical and/or physical interactions between the compounds and the packing particles. Finally, the separated compounds are detected at the exit of the column by a detector that measures their amount. The HPLC procedures with appropriate detection system are considered as the most convenient methods for the determination of B vitamins (Lebiedzinska et al., 2007).

During the past two decades, different HPLC methods have been developed and reported in the literature for vitamins analysis (Blake, 2007). However, most of these methods only focus on the analyses of a single vitamin at a time. With the current improvement in the chromatography technology, many procedures allowing the analysis of multivitamins in various food matrices have been proposed (table 7). Major improvement in the field of HPLC for water soluble vitamins analysis, have been performed regarding the separation and the detection of these compounds.

Table 7. Example of published multivitamin method of analysis by liquid chromatography (LC)

Vitamins analysed	Food products	LC procedures	Reference
Thiamin, riboflavin, nicotinamide, nicotinic acid, pantothenic acid, pyridoxal,pyridoxine	Rye flours, white and red rye malt, yeast, rye sourdough breads	LC on C-18 1.8 µm (2.1 x 150 mm) column, eluted with a gradient of water (0.1 % formic acid) and acetonitrile (0.1% formic acid) **Detection**: LCT Premier^{TM}XE ESI TOF MS system in positive ion mode	Mihhalevski et al., 2013
Thiamin, riboflavin, nicotinamide, nicotinic acid,pyridoxal, folic acid, ascorbic acid	Breakfast cereal matrix	LC on HILIC column eluted with a gradient of ammonium acetate in acetonitrile and water **Detection**: UV: Thiamine, nicotinic acid, nicotinamide, folic acid; Fluorimetric: Riiboflavin, pyridoxal; Coulometric: pyridoxal, folic acid, ascorbic acid	Langer and Lodge, 2014

Vitamins analysed	Food products	LC procedures	Reference
Thiamin, riboflavin, nicotinic acid, nicotinamide, pantothenic acid, biotin, folic acid, cobalamin, ascorbic acid, pyridoxamine, pyridoxamine 5P, pyridoxal 5P, pyridoxal, pyridoxine	Maize flour, green and golden kiwi, tomato pulp	LC on C-18 (25cm x 4.6 mm i.d.; 5 µm)column, eluted with a gradient of acetonitrile and water both acidified with formic acid **Detection**: PE Sciex API 3000 triple-quadrupole mass spectrometer in positive ion mode	Gentili et al., 2008
Thiamin, pyridoxamine, pyridoxal, pyridoxine, cobalamin	Plant foods and animal foods	LC on C-18 5 µm (25cm x 4.6 mm) column, eluted with phosphate buffer, methanol (90:10) and trimethylamine **Detection**: UV: Thiamine; Electrochemical: pyridoxamine, pyridoxal, pyridoxine, cobalamin);	Lebiedzinska et al., 2007
Thiamin, riboflavin, nicotinamide, nicotinic acid,pyridoxal,pyridoxine folic acid, ascorbic acid, pantothenic acid	Multivitamin tablets	LC on C-18 (250mm x 4.6mm) column, eluted with a gradient of aqueous solution of heptafluorobutyric acid and methanol **Detection** : Micromass ZQ 2000 spectrometer simple quadrupole in positive ion mode	Chen et al., 2006
Thiamin, riboflavin, niacin, pantothenic acid, pyridoxine, folic acid, cyanocobalamin, ascorbic acid	Enriched milk and infant nutrition products	LC on C-18 ODS-2 (25 cm x 4.6 mm, 3 µm) column, eluted with a gradient of buffer phosphate and methanol **Detection**: UV: Thiamin, pantothenic acid, folic acid, ascorbic acid, cyanocobalamin; Fluorimetric: pyridoxine, riboflavin	Zafra-Gomez et al., 2006

Vitamins analysed	Food products	LC procedures	Reference
Thiamin, riboflavin, niacin, pantothenic acid, pyridoxine, folic acid, ascorbic acid	Tarhana (Traditional turkish cereal food)	LC on C-18 (150mm x 4.6mm) column, eluted with KH_2PO_4-methanol (90:10) in isocratic mode **Detection**: UV	Ekinci and Kadaka, 2005
Thiamin, pyridoxine, pyridoxal, pyridoxamine, nicotinamide, nicotinic acid, pantothenic acid, folic acid	Durum wheat flour, dough and pasta samples	LC on C-16 amide (15cm x 4.6mm, 5 µm) column, eluted with a gradient of ammonium formate and methanol **Detection**: PE-Sciex API 365 triple-quadrupole mass spectrometer, in positive and negative ion mode	Leporati et al., 2005
Riboflavin, pyridoxine, thiamin, ascorbic acid	Human urine	LC on C-18 (250 x 4.6mm, 5 µm) column, eluted with a gradient of 0.1% TFA containing water and 0.1% TFA containing 90:10 methanol/water **Detection**: UV	Cho et al., 2005

Enhancing separation of water-soluble vitamins using new columns

The broad range of polarity found within the water soluble vitamins rendered a very tough judgment about the choice of column.

Adsorption chromatography depends on the chemical interaction between solute molecules and specifically designed ligands chemically grafted to a chromatography matrix. Over the years, different ligands have been developed to improve the separation of a large set of biomolecules. An important contribution to the range of adsorption techniques for preparative chromatography of biomolecules has been reversed phase chromatography in which the binding of mobile phase solute to an immobilized n-alkyl hydrocarbon or aromatic ligand occurs via hydrophobic interaction. Reverse phase chromatography has found analytical applications in the area of water-soluble vitamins separation (Vinas et al., 2003; Heudi et al., 2005). Though, some analytes, especially thiamin and pyridoxine are ionic compounds that are not well retained in reversed-phase chromatography. Therefore reported methods in literature for these vitamins usually involve the use of ion-exchange chromatography (Callmer and Davies, 1974) or of ion pairing reagent for reversed-phase chromatography to enhance their retention on the

column (Albala-Hurtado et al., 1997; Almagro et al., 2002; Zafra-Gomez et al., 2006). However, the biggest drawback of this method is that the ion-pair reagents are hard to be fully washed from the column, which requires the dedication of a particular column to ion-pair applications (LoBrutto et al., 2005). Other improvements of the retention properties of reversed phase chromatography have emerged during the past decade, through the development of polar-embedded and polar-endcapped column (Wilson et al., 2004; Layne, 2002). These phases involve modifications of the chemistry of classical alkyl phases through either an insertion of a polar functional group (amide, urea, carbamate and ether groups) within the alkyl chain attached to the silica surface for the former or the deactivation of residual silanols with polar functional group (amino or hydroxyl terminated short alkyl chain) for the latter (Layne, 2002).

Recently, the hydrophilic interaction liquid chromatography (HILIC) has been developed in order to overcome the problem of highly polar compounds that could not be ionized in solution and as a consequence could not be retained by either ion-exchange chromatography or ion pairing on reverse phase column. The term “hydrophilic” refers to the affinity to water. Typical components of HILIC mobile phase include a high percentage of organic solvent with water and buffer as the modifier. Conceptually, the aqueous portion in the mobile phase is preferentially adsorbed onto the polar stationary phase, establishing a water-enriched layer. This semi-immobilized polar layer is sandwiched between the stationary phase surface and the organic-solvent rich mobile phase. It is the partitioning of the analytes between these two layers that result in the retention and separation in HILIC. Originally, HILIC was applied mainly for the determination of carbohydrates, amino acids and peptides, but the interest in the HILIC technique in the last years has been promoted by growing demands for the analysis of polar drugs metabolites and biologically important compounds (Strege, 1998). The HILIC has also been shown to successfully achieve the separation of six water-soluble vitamins (Langer and lodge, 2014; Karatapanis et al., 2009). Another reason for the increasing popularity of HILIC is its excellent suitability for coupling to mass spectrometry (Kahsay et al., 2014).

Multivitamin analysis using MS detector

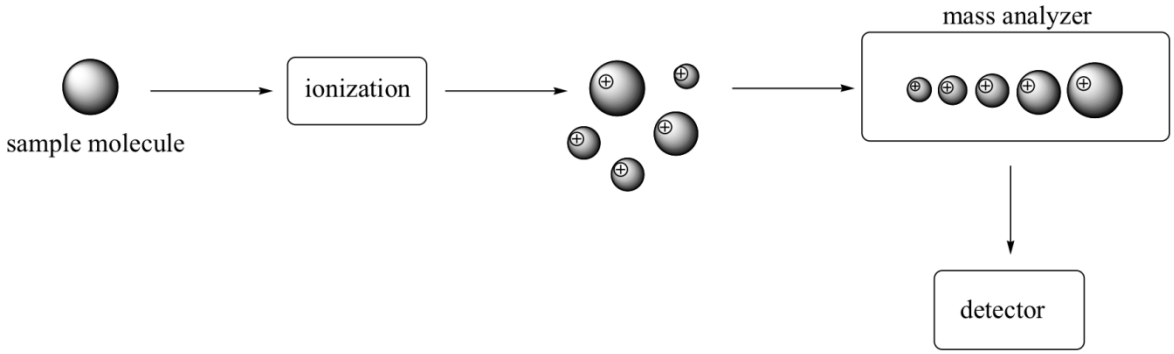
Commonly, the HPLC procedures have been coupled with fluorescence detector and/or diode array detector (DAD) for the determination of B vitamins (Batifoulier et al., 2005; Ekinci and Kadakal, 2005; Cho et al., 2000; Gratacos-Cubarsi et al., 2011; Zafra-Gomez, 2006). However, detection at low wavelengths required for non-chromophoric vitamins like pantothenic acid is possibly subject to background interferences and noisy baseline (Trang, 2013). In addition fluorescence detection is useful only for those vitamins that are naturally fluorescent (Vitamin B2 and B6) or become fluorescent after derivatization. Lebiedzinska et al. (2007) used coulometric electrochemical and ultraviolet detection to the simultaneous determination of vitamins B1, B6 and

B12 in fortified food product. Trang (2013) in his HPLC method using evaporative light scattering (ELSD) as a detector, demonstrated that ELSD has a much lower sensitivity than diode array detector for multivitamin analysis of pharmaceuticals. Nowadays, numerous publications describing the use of LC procedures with mass spectrometry detection have been reviewed (Leporati et al., 2005; Vazquez et al., 2009; Gentilli et al., 2008; Lu et al., 2008; Chen et al., 2006; Mihhalevski et al., 2013).

Mass spectrometry is an analytical technique that measures the molecular masses of individual compounds and atoms precisely by converting them into charged ions. There are three basic steps involved in mass spectrometry analysis (figure 28).

1. The first step is ionization that converts analyte molecules or atoms into gas-phase ionic species. This step requires the removal or addition of an electron or proton(s).
2. The second step is the separation and mass analysis of the molecular ions and their charged fragments on the basis of their m/z (masse-to-charge) ratios.
3. Finally, the separated ions are measured, amplified, and displayed in the form of a mass spectrum.

Figure 28. Basic concept of mass spectrometry analysis



There are many different types of MS instruments, but they all have the same three essential components. First there is an ionization source that converts the neutral sample molecules into gas-phase ions. Several ionization techniques have been developed for this purpose. Depending on the ionization method used, the ionized molecule may or may not break into a population of smaller fragments. In the figure 28, some of the sample molecules remain whole, while others fragment into smaller pieces. Next in line there is a mass analyzer that separates and mass-analyzes the ionic species. Magnetic and/or electric fields are used in mass analyzers to control the motion of ions. A magnetic sector, quadrupole, time-of-flight, quadrupole ion trap, orbitrap and Fourier transform ion cyclotron resonance instrument are the most common forms of mass analyzers currently in use. Finally, there is a detector which measures and amplifies the ion current of mass-resolved ions.

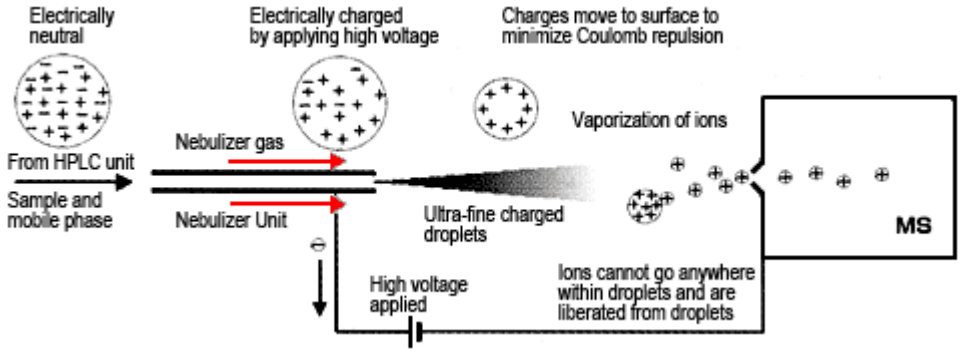
Modes of ionization

The function of an ion source is to convert a neutral compound to a gas-phase ionic species. There are a plethora of ion sources available, but soft ionization seemed preferable for vitamins analysis.

Electrospray ionization (ESI).

This ionization method is a soft ionization process which has become the most successful interface for LS/MS applications (Lu et al., 2008; Chen et al., 2006; Vasquez et al., 2009; Mihhalevski et al., 2013). Electrospray ionization is a process that produces a fine spray of highly charged droplets under the influence of an intense electric field. Evaporation of the solvent converts those charged droplets into gas-phase ions. A simplistic view of the ESI process is depicted schematically in figure 29. Electrospray analysis can be performed in positive and negative ionization modes. Leporati et al. (2005), demonstrated that in general, water-soluble vitamins respond better in positive ionization mode with an exception for folic and ascorbic acid which respond better in the negative ionization (Trang, 2013) . The mechanism of ESI is a highly debated topic, however it is generally believed that ionization in electrospray involves three different processes: droplet formation, droplet shrinkage, and desorption of gaseous ions. Two mechanisms have been proposed to explain ion desorption from the droplets: The ion-desorption model (IDM) and the charge-residue model (CRM). The IDM suggests that as the droplet reaches a certain radius, the field strength at the surface of the droplet becomes large enough to assist the field desorption of solvated ions (Nguyen, 2007). The CRM suggests that electrospray droplets undergo evaporation and fission cycles, eventually leading progeny droplets that contain on average one analyte ion or less (Dole et al., 1968). The coupling of an ESI source with a quadrupole analyzer is the most successful ESI-MS combination.

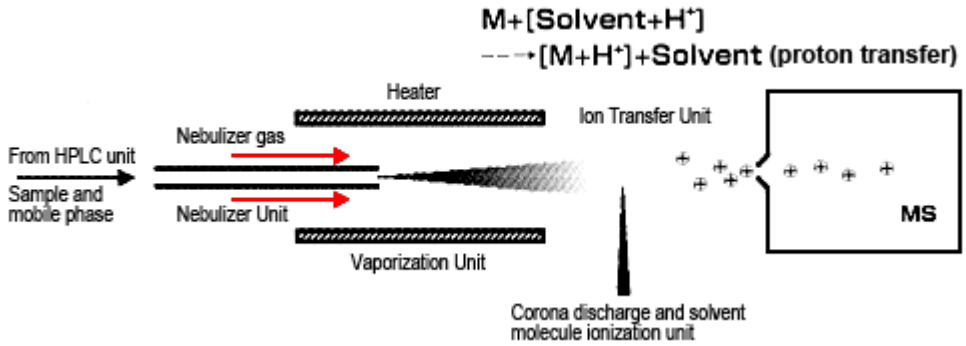
Figure 29. Evaporation of ions in Electrospray Ionization (ESI)(sources from Shimadzu, analytical and measuring instruments)



Atmospheric pressure chemical ionization (APCI).

APCI is an ionization method relies upon the formation of a plasma of ions comprised, mainly, from the HPLC mobile phase components. The eluent molecules, which are in vast excess compared to the analyte, are ionized by the electron cloud around the corona discharged (figure 30) pin and act as the reagent gas in a chemical ionization process. Generally, the primary ions formed by the corona discharged are ions such as positively charged nitrogen or oxygen radical which can then form secondary reactant gas ions though collision with vaporized solvent molecules. APCI considerably reduces the thermal decomposition of the analyte because of the rapid desolvation and vaporization of the droplets in the initial stages of the ionization. Thus, this ionization mode is very soft and rarely produces major fragmentation of the analyte ion. Unlike in ESI, the solvent-evaporation processes are separated in APCI, this allows the use of low-polarity solvents that are unfavorable for ESI ion formation. Another advantage to use APCI over other ionization methods is that it allows for the high flow rates (1mL/min and higher) to be used directly. However, as mentioned by Leporati et al.(2005) even if ESI and APCI ionization techniques demonstrated to be extremely powerful for the determination of water-soluble vitamins in foods, ESI seemed to be superior as it permitted the analysis of nine B-vitamins (including folic acid) and presented better inter-day precision. APCI is suitable for compounds ranging from low to medium polarities, which include also carotenoids and related compounds. Lanina et al. (2007) have evaluated the ESI and APCI ionization techniques in both positive and negative ion modes for the simultaneous LC-MS analysis of the four tocopherol homologues and demonstrated that the APCI in negative ion mode was more efficient.

Figure 30. Ion-molecule reactions in Atmospheric Pressure Chemical Ionization (APCI) (sources from Shimadzu, analytical and measuring instruments)



Mass analyzers

Mass analyzers separate the ions according to their mass-to-charge ratio. All commonly used mass analyzers use electric and magnetic fields to apply a force on charged particles (ions). The choice of mass analyzer is based upon the application, cost and performance desired. There is no ideal mass analyzer that is good for all applications

Time-of-flight (TOF) mass analyzers

The time-of-flight mass spectrometer uses an electric field to accelerate the ions through the same potential, and then measures the time they take to reach the detector. If all the particles have the same charge, the velocity will be dependent on their mass-to-charge ratio. Lighter ions will reach the detector first. TOF mass analyzers are well suited for obtaining mass spectra of gas chromatography effluents.

Quadrupole mass analyzers

The quadrupole mass analyzer is a “mass filter”. It consists of four cylindrical rods, set parallel to each other. Each opposing rod pair is connected together, and a radio frequency (RF) voltage is applied between one pair of rods and the other. Only ions of a certain mass-to-charge ratio will reach the detector for a given ratio of voltages: other ions have unstable trajectories and will collide with the rods. This permits selection of an ion with a particular m/z or allows the operator to scan for a range of m/z values by continuously varying the applied voltage. These mass spectrometers are very useful in liquid chromatography-mass spectrometry or gas chromatography-mass spectrometry where they serve as high specific detectors for particular ions of interest.

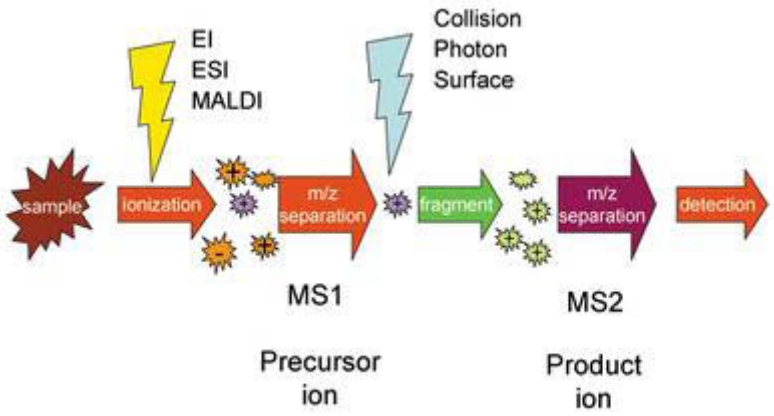
Trapped-ions mass analyzers

There are two principal trapped-ions mass analyzers: three dimensional or linear quadrupole ion traps ("dynamic traps") and ion cyclotron resonance mass spectrometers ("static" traps). Both operate by storing ions in the trap and manipulating the ions by using DC and RF electric fields in a series of carefully timed events. Metabolomic is a field that benefits from the accurate mass measurement and high resolving power MS/MS features of the Linear Trap Quadrupole-Orbitrap (Perry et al., 2008).

Tandem mass spectrometry

Tandem mass spectrometry, also known as MS/MS or MS^2, involves multiple steps of mass spectrometry selection with fragmentation occurring between stages (Figure 31). In the most common tandem mass spectrometry experiment a first analyzer is used to isolate the precursor ion, which then undergoes fragmentation to yield a products ion and neutral fragments which are then analyzed by a second mass analyzer. This experiment is commonly performed to identify transitions used for quantification by tandem MS. There are two main categories of instruments that allow tandem mass spectrometry experiments: tandem mass spectrometry in space or in time.

Figure 31. Schematic of tandem mass spectrometry



As mentioned by Leporati et al. (2005), most of the water-soluble vitamins are low-molecular-weight compounds, and their determination by single quadrupole MS could be affected by matrix interferences. In addition, since the levels of the vitamins are rather low in food products, the high sensitivity and specificity of tandem mass spectrometry (MS/MS) have been used to develop methods for the determination of vitamins content in different foodstuffs (Leporati et al., 2005; Gentili et al., 2008) or in fortified infant foods (Lu et al., 2008).

5.2.2. The Fat-soluble vitamins and carotenoids

5.2.2.1. Extraction methods

The procedure applied to food products for carotenoids extraction begins with an extraction with methanol (MeOH) or a mixture of MeOH and other more apolar dissolvents. Hart and Scott (1995), for example, used a MeOH-tetrahydrofuran (THF) (1:1, v/v) solution in the carotenoid analysis of a wide variety of vegetables and fruits, both raw and cooked. MeOH and tetrahydrofuran (Burkhard and Böhm, 2007), MeOH and hexane (Schmitz et al., 1989). Other groups prefer the use of n-hexane/ethyl acetate (9:1 v/v) or with water-saturated n-butyl alcohol (WSB) (Panfili et al., 2004). Extraction can also be performed using solid phase extraction (SPE) (Irakli et al., 2011) or supercritical fluid extraction (Nakornriab et al., 2008). Vitamin E can be extracted from cereal samples either by a mixture of ethanol (in order to remove the proteins) and *n*-hexane/ ethyl acetate or by methanol. Carotenoids and vitamin E in vegetables and fruits are predominantly esterified by fatty acids. Procedures such as saponification in an alkaline environment which hydrolyze lipid esters to more hydrophilic fatty acids and alcohols are needed. In addition as mentioned by Irakli et al. (2011), carotenoids are often bound to proteins, in the form of complexes known as carotenoproteins. Thus, saponification aims to favor the release of carotenoids from these complexes. However, depending on the nature of the carotenoids and the food type (Olivier et al., 1998), saponification may result in destruction or structural transformation. Procedures using antioxidants, nitrogen flushing or involving limited exposure to light and oxygen or rapid cooling of the reaction mixture after the saponification have shown to be efficient for the complete extraction of vitamin E and carotenoids in cereal samples (Panfili et al., 2004; Irakli et al., 2011). Hidalgo et al. (2006), compared protocols for carotenoids extraction (WSB, the hot saponification and tetrahydrofuran) and the protocols for tocols extraction (WSB, hot-saponification, methanol and room temperature saponification) on the same einkorn sample. They concluded that the butanol method extracted significantly better carotenoids than the two other techniques, whereas in the case of tocols, the hot saponification has better extraction efficiency than the others.

5.2.2.2. Methods of analysis

Historically, most of the carotene data in tables of food composition have been obtained by measuring total absorption at a specific wavelength and quantified against β-carotene. The evaluation of the color of durum semolina and pasta are using visual comparison with standard samples, light reflectance measurement (colorimetric) and spectrophotometrical determination after chemical pigment extraction. In recent years, high-performance liquid chromatography has become the method of choice for the analysis of carotenoids because of its ability to distinguish between similar geometrical structures of carotenoids. Numerous methods for determination of vitamin E are available: titrometric, or-

currently most frequently used- instrumental: electrochemical, fluorometric, spectrophotometric (Rutkowsky and Grzegorczyk, 2007). Such methods are interesting for the total quantification of vitamin E in samples but do not allow to independently analyzing tocopherols and tocotrienols. HPLC provides a convenient method for quantification and characterization purposes of tocopherols and tocotrienols.

Chromatographic procedures

Based on their chemical properties, tocopherol and tocotrienol can be separated by using either normal or reversed-phase column. Although reversed-phase systems are more widely used for routine analysis as they have better stability and longer durability than normal phase column (Irakli et al., 2011). Considering the qualitative and quantitative variability of tocopherols and tocotrienols in plants, the use of normal phase columns is required for the food samples analysis, like some cereals such as barley, which contain all of the vitamin E vitamers, including β and ϒ isomers of tocopherols and tocotrienols (Panfili et al., 2003). In general, both normal-phase and reverse-phase HPLC have shown the capability to separate closely related carotenoid pigments. However, the most frequently used method for separating carotenoids in foods is reversed-phase HPLC (Irakli et al., 2011). Detection mode involved for the determination of tocols include UV, photodiode array, fluorimetric electrochemical as well as MS detection (Vasquez et al., 2009; Hao et al., 2005; Lanina et al., 2007). Carotenoids are generally detected with photodiode array detector (Panfili et al., 2004; Leenhardt et al., 2006; Hidalgo et al., 2006).

The use of MS detection is a powerful tool for the identification of carotenoids, when it is coupled with LC, to exploit the chromatographic separation of isomers, e.g. zeaxanthin and lutein.

Enhancing separation of fat-soluble vitamins and carotenoids using new columns

The introduction of new stationary phases capable of separating β and γ isomers under reversed-phase HPLC conditions, e.g. long-chain alkyl-bonded C_{30}-silica has been successfully applied to the separation of these isomers (Sander et al., 2000). The C_{30} columns provide longer retention times and greater shape selectivity compared to C_{18} phases due to their rigid highly ordered C_{30} alkyl groups. The C_{30} column also provided an excellent separation for carotenoid stereo-isomers (Burkhardt and Böhm, 2007). However, its extended running time up to 80 min without equilibration time limited its application in practice (Sander et al., 2000).

Multivitamin analysis using MS detector

Few HPLC methods have been developed for simultaneous determination of fat-soluble vitamins. Irakli et al. (2011) have developed a reversed-phase method

for the determination of three tocopherols, three tocotrienols and two carotenoids in cereals. In the work presented by Li and Chen (2001), twelve water- and fat-soluble vitamins were simultaneously determined in pharmaceutical preparations using a gradient HPLC with diode array detection. The mass spectrometry technique has not been widely used for the analysis of vitamin E owing to the difficulty involved in the ionization of non-polar molecules (Bastamante-Rangel, 2007). Hao et al. (2005) have developed a rapid and sensitive method for the simultaneous quantification of α-tocopherol, β-carotene, β-cryptoxanthin, lutein and zeaxanthin in botanical materials using normal phase liquid chromatography-atmospheric pressure chemical ionization-tandem mass spectrometry. They observed that the sensitivity of the method was improved when APCI ionization technique in positive ion mode was applied. However, in the method developed by Lanina et al. (2007), the APCI ionization in negative ion mode was the more efficient technique for simultaneous analysis of the four tocopherol homologues. On the other hand, Bastamante-Rangel et al. (2007) have shown that the addition of a base to the mobile phase was required to enhance the ionization of tocopherols and tocotrienols in negative ion mode electrospray ionization.

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Objectives of the thesis

Wheat is by far the most important crop for breadmaking because of its supreme baking performance in comparison with all other cereals. However, as shown in literature, the nutritional quality of bread wheat is affected by processing such as milling, mixing, fermentation and baking. In addition, the composition of bread wheat in micronutrients is influenced by genotype, by environment and/or by genotype x environment interactions. The efficiency of wheat selection, grinding processes or industrial processing might be a key to enhance the nutritional value of wheat-food products. However, to this aim, the development of high throughput methods determining the composition of vitamins within the different wheat fractions or end-use products has been explored. Nevertheless, in the objective of a large scale varietal screening, none of the reported procedures are suitable for the analysis of wheat flour and wheat food products. Despite the potential of liquid chromatography/tandem mass spectrometry (LC-MS/MS), only a few methods have been applied for the determination of multivitamin in food samples. Thus, the first objective was:

- To develop and validate a rapid, high throughput LC-MS/MS methods for the determination of B and E vitamins, Lutein and β-sitosterol in various wheat-based food materials.

Nowadays the nutritional value of bread as affected by ingredients, storage, processing techniques (milling technologies and bread making) are well reviewed and tend to show the ways by which the nutritional quality of wheat product can be improved to maintain the potential health benefits of whole-grain wheat. The aim of the more recent milling process is to produce new fractions with enhanced nutritional quality. However, because of the lack of efficient and rapid biochemical analytic methods, little investigation has been conducted on the composition in bioactive compounds of the different wheat milling fractions produced during the various processing operations. In addition, the effect of breadmaking process of toasted bread made with enriched wheat flour has never been examined. Thus, using the method developed, the second objective was:

- To study the concentration of B and E vitamins, Lutein and β-sitosterol of the different wheat milling fractions produced with an innovative milling process.
- To compare the susceptibility of vitamin content (thiamin, riboflavin, nicotinic acid, nicotinamide, pantothenic acid, pyridoxine, pyridoxal, tocopherols (alpha-tocopherol, gamma-tocopherol), tocotrienols (alpha-tocotrienol, beta, gamma-tocotrienol), lutein and beta-sitosterol) at each step of the breadmaking process of toasted bread produced with semi coarse wheat flours (type 110) obtained from a bakery company.

The HEALTHGRAIN study (2005-2010), supported by the EU under Framework 6, has generated the largest database currently available on the contents of bioactive components in wheat. These include a wide diversity survey of 150 bread wheat lines grown on a single site (Ward et al., 2008) and a more detailed study of 26 lines (Shewry et al., 2010; Shewry et al., 2011). However, these studies were conducted on a limited range of bioactive components as it was not possible to measure the entire metabolite content using a single analytical technique (Shewry et al., 2012). Thus, the third objective was:

- To quantify and analysed numerous agronomic and quality traits as well as the content of Lutein, β-sitosterol, fat and water-soluble vitamins in a core collection of hexaploid wheat grown in two environments.

In order to develop genetic markers to help breeders to select lines with enhanced nutritional value, it is necessary to identify the genes responsible for the traits, or at least to determine their precise locations on a genetic map (Shewry et al., 2012). With recent developments in statistics and high throughput genotyping, association analysis can now be applied to many different kinds of populations, such as genetic resource collections, for mapping regions responsible for the variation in many traits (Bordes et al., 2011). Thus, the last objective of this thesis was:

To scan the genome for new loci involved in the genetic control of wheat vitamins composition by association mapping in a core collection of 196 accessions genotyped with DArT, SSR and SNP markers on the wide genome

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Materials and Methods

1. Development of a LC-MS/MS method for the simultaneous screening of 7 water-soluble vitamins in processing semi-coarse wheat flour products.

1.1. Chemicals and Reagents

Thiamin hydrochloride, riboflavin, nicotinamide, pyridoxine hydrochloride, pyridoxal hydrochloride, nicotinic acid, pantothenic acid, ethanol absolute, acetonitrile, glyoxylic acid, sodium acetate, L-glutathione reduced, Ethylene-diamine-tetra-acetic acid, Acid phosphatase from potato, taka-diastase from Aspergillus oryzae, beta-glucosidase were obtained from Sigma Aldrich (Saint Quentin Fallavier, France). All chemical were of analytical-reagent grade (purity>98%). Papain and iron (II) sulfate heptahydrate was purchased from Merck. Thiamin-d3-hydrochloride, pantothenic acid-13C3, 15N hemicalcium salt and nicotinamide-d4 were obtained from Toronto Research Chemicals. For LC analysis, distilled water was further purified by passing it through a Milli-Q plus apparatus (Millipore, corporation France).

Different stock and standard solutions were prepared:

- Internal standard stock solutions were prepared by dissolving one milligram of each labeled internal standard (thiamin-d3-hydrochloride, pantothenic acid-13C3, 15N hemicalcium salt and nicotinamide-d4) in 1 mL of acetonitrile/water (50/50). They were stable for at least 1 month when stored in the dark at +4°C.
- Mix internal standard solution was prepared prior to sample extraction by mixing and diluting individual internal standard stock solutions in acetonitrile/water (50/50) to obtain a concentration of (15.6 µg/mL) for all labeled standard.
- Unlabeled external standard stock solutions (approximately 2 mg/mL) were prepared in acetonitrile/water (50/50) and were stable for at least 1 month when stored in the dark at +4°C.

1.2. Wheat food samples

Eight semi-coarse wheat (***Triticum aestivum L***) flours ("type 110"), eight bread making doughs, eight semi-coarse breads and eight semi-coarse toasted breads (See in Material and Methods **2. Change in E vitamin and Lutein, β-sitosterol contents in industrial milling fractions and during toasted bread production**, were directly taken from an industrial process and immediately stored at -80°C. Dough and bread were freeze-dried before analysis. The extraction of the vitamins in the different matrix was performed according to the method of Ndaw et al (2000).
The moisture content of flour and toasted bread was determined using the difference between wet mass and dry mass on sample aliquots that were dried at constant temperature.

1.3. Extraction Procedure with enzymatic hydrolysis

625 mg of sample was transferred into a 50-mL FALCON tube. 100 µL of mix internal standard solution (15.6 µg/mL) was then added. 4.75 mL of sodium acetate (pH 4.5) was added to the sample. 1.25 mL of 0.5M glyoxylic acid solution, 0.25 mL of 1 % L-glutathione reduced solution, 0.25 mL of 1% ethylene-diamine-tetra-acetic acid solution and 0.2 ml of 2% iron (II) sulfate heptahydrate solution were added to the sample. One mL of a mixture of enzyme solution in sodium acetate (pH 4.5) containing 2.5 mg of phosphatase, 12.5 mg of papain, 62.5 mg of takadiastase and 2.5 mg of β-glucosidase was added and, the mixture was strongly mixed and incubated at 37°C for 14 h. The cooled sample was vortexed and centrifuged at 12,000 x g for 10 min. The supernatant was filtered through a 0.22 µm Millex filter (Milipore, MA, USA). 10 µL of the filtrate was injected in duplicate into the LC-MS/MS system.

1.4. Extraction Procedure without enzymatic hydrolysis

Same protocol as above but without the addition of the mixture of enzyme.

1.5. LC-(ESI)MS/MS analysis

Analytes were detected and quantified using a liquid chromatography Agilent 1100 HPLC system equipped with a binary pump, autosampler and a Diode array multiple wavelength detectors (Agilent Technologies, Palo Alto, CA, USA) and coupled to a triple quadrupole mass spectrometer (MS) API 2000 (Applied Biosystems/MDS Sciex, Foster City, CA, USA), equipped with an electrospray ionization source interface operating in positive mode.
MS parameters were optimized by direct infusion of standard solutions. Maximum sensitivity was obtained for a capillary voltage at 5000 V, a source temperature at 400 °C, and GS1 and GS2 at 30 and 50 respectively. The source parameters were optimized for each standard to obtain the best sensitivity.
Chromatographic separation of the analytes was achieved on different columns. A Waters (Saint Quentin en Yvelines, France) Atlantis dC18 column (2.1 mmx150 mm, 5µm) and a Nucleodur Polar tech (Macherey-Nagel, EC 150/2,5um). Both columns were eluted using the same chromatographic conditions. Aqueous mobile phase (phase A) and methanol (phase B) were used and acidified with 0.1% formic acid to promote electrospray ionization of the basic functions of all vitamins. The flow rate of the mobile phase was 0.3 mL/min. The elution gradient was the following: A/B 100/0 (v/v), hold for 1 min; from 0 to 100% B in 4 min (linear gradient); 100% B hold for 5 min, then increased to 100% A in 0.1min and hold for 5min to the next injection. The column effluent was connected to the valco valve of the mass spectrometer and first minute of elution was splited to waste. Optimization of the instrumental parameters was performed by infusing standard solutions (10 µg/mL) of the analytes in a 50/50 water/acetonitrile mixture.

1.6. Matrix Effects

Two sets of samples containing the three labeled standards were prepared to evaluate the absence or presence of matrix effect. The first set (1) was prepared by mixing thiamin-d3-hydrochloride, pantothenic acid-$^{13}C3$, ^{15}N hemicalcium salt and nicotinamide-d4 in 50/50

water/acetonitrile mixture at the concentration of 0.2 µg/mL for each analyte. The second set (2) was prepared as follows: The four different biological materials were first extracted and spiked after extraction with the three labeled standards at the concentration of 0.2 µg/mL. 10 µL of each set was injected into the LC-MS/MS system. The ion suppression or enhancement was determined by comparing the absolute peak areas of each of the labeled compounds in the set (1) and (2).

1.7. Quantitative analysis

Quantification was achieved by measuring product ions (multiple reaction monitoring [MRM]) from the fragmentation of the protonated [M + H]+ molecules. The collision energy potential and the collision exit potential were then adjusted to optimize the signal for the most abundant product (daughter) ions.

Concentration of each vitamin in the sample was calculated using 7-point calibration curves ranging from 50ng/mL to 9µg/mL. Calibration curve standards were prepared by adding 100µL aliquots of mix unlabeled external standards solution with varying concentrations (0.25-45 µg/mL) into Eppendorf tubes containing 100 µL of thiamin-d3-hydrochloride (1 µg/mL), 100 µL of pantothenic acid-$^{13}C3,^{15}N$ (1 µg/mL), 100 µL of nicotinamide-d4 (1 µg/mL) and 100 µL of acetonitrile/water (50/50).

The calibration curves were constructed by relating the varying concentrations of unlabeled external standards to their relative response factors as determined by the ratio of the peak intensity of the unlabeled external standards to that of the corresponding labeled internal standard. The most important LC/ESI-MS parameters for MRM acquisition of the 7 target compounds are summarized in (Table 1).

1.8. Validation of the method

Precision and bias of the method were determined using the developed method: The sample was treated using the extraction method and analyzed by LC-MS/MS as described above in materials and methods.

The linearity of the calibration curve was evaluated using 35 determinations (5 repetitions at 7 concentration levels) for each analyte within the range 0.05 to 9 µg/mL and estimated with an F-test analysis (p=0.01).

The Intra-day precision was evaluated for each analyte in each matrix by preparing and analyzing 6 times one sample in one day (the sample was injected 4 times in the LC-MS/MS system).The relative standard deviations (RSDs) from the 24 measurements were examined.

The Inter-day precision was estimated for each analyte in each matrix by preparing and analyzing one sample on each of 6 consecutive days (each day the sample was injected 4 times in the LC-MS-MS system). The RSDs from the 24 measurements performed were examined.

Method bias was validated using a spiked-recovery test. Spiked at five levels (1.25, 1.56, 2.08, 6.25, 12.5 µg) were used for each vitamins in each food sample before extraction. Two determinations were carried out for each addition level. In order to determine if bias was acceptable a linear regression approach was tested and a Student t-Test was performed.

Table1. LC-MS/MS parameters for the detection of water-soluble vitamins

Analyte	Retention time (min)	MRM[a] (m/z)	Lens parameters					
			Declustering Potential (V)	Focusing Potential (V)	Entrance Potential (V)	Cell Entrance Potential (V)	Collision Energy (V)	Cell Exit Potential (V)
Thiamin	1.39	265/122	11	300	4.5	20	23	2
Thiamin-d3	1.39	268/125	16	290	6	18	21	4
Pyridoxal	1.69	168/150	11	270	7.5	16	17	2
Pyridoxine	1.83	170/152	21	340	6	14	19	2
Nicotinic acid	1.90	124/80	26	300	12	10	27	4
Nicotinamide	1.94	123/80	31	320	5	12	27	0
Nicotinamide-d4	1.94	127/84	22	300	19	14	33	0
Pantothenic acid	7.29	220/90	21	330	10.5	16	21	0
Pantothenic Acid-$^{13}C3,^{15}N$	7.29	224/94	26	370	11.5	20	19	0
Riboflavin	8.34	377/243	21	270	8	32	35	4

[a]MRM : Multiple reaction monitoring

1.9. Statistical analysis

During the experiment using two different extraction conditions, only one sample from the eight available in wheat flour, in bread and in toasted bread were analyzed in duplicate for B vitamin content.
Results for B vitamin levels in each food matrice correspond to the mean of duplicate samples of the 8 wheat flours, doughs, breads and toasted breads.
Data were analyzed using analysis of variance (ANOVA) and differences in the vitamin contents were evaluated using a Fishers Least Significant Difference (LSD) test.

Ndaw, S., Bergaentzlé ,M., Aoudé-Werner, .D, Hasselmann, C *.2000. Extraction procedures for the liquid chromatographic determination of thiamin, riboflavin and vitamin B6 in foodstuffs. Food Chemistry 71, 129-138.*

2. Change in E vitamin and Lutein, β-sitosterol contents in industrial milling fractions and during toasted bread production.

2.1. Selection and milling of wheat cultivar

Two common wheat cultivars (Renan and Astardo), collected from two private cereal collectors, were selected because of their good rheological properties and their availability from the suppliers, in order to investigate the variability in vitamin content. Whole grains of these two varieties were milled and blended by an industrial milling company for satisfying rheological and technological uses and two different industrial processes of milling (conventional and improved) were used. For conventional milling, five milled fractions were obtained: white flour, 77%; coarse bran, 10%; thin bran, 8%; brown middling, 2%; white middling, 3%. For Improved process, two other milled fractions were studied, discard sifting and enriched fraction.

Semi-coarse wheat flour type of 110, obtained from industrial milling company and milling fractions were stored a -80°C before vitamin extraction and analysis.
To monitor the efficiency of the milling process upon the production of the enriched fraction, we determined the nutritional composition of the generated seven milling fractions.

2.2. Toasted bread preparation

Breads were produced with the constituted semi-coarse wheat flours according to the industrial process developed by a bakery industry. Briefly, all breads were prepared in accordance with the normalised bread making process AFNOR V03-716 procedure and osmotolerant yeasts (Mauri Extra OSMO AB Mauri, France) were used for dough fermentation. Two steps of fermentation were allowed: first fermentation 10min at 25°C, second fermentation 50min at 38°C. Baking was performed at 270°C for 25 min. Bread was

then carried out at low temperature 20°C over 10 h. The stale bread was sliced in uniform thickness parts and toasted for 21 min at 200°C.
For analytical determinations, duplicate samples of unleavened dough (just after kneading), whole wheat bread and toasted bread were collected and immediately stored at -80°C. Dough and bread were freeze-dried before analysis. They were finely grounded before extraction of B and fat-soluble vitamins.
The moisture content of flour and toasted bread was determined using the difference between wet mass and dry mass on sample aliquots that were dried at constant temperature.

2.3. Analytical methods

2.3.1. Instrumentation

Analytes were detected and quantified using a liquid chromatography Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA) coupled to a triple quadrupole mass spectrometer (MS) API 2000 (Applied Biosystems/MDS Sciex, Foster City, CA, USA) equipped with an atmospheric pressure chemical ionization or an electrospray ionization interfaces. Both sources were operated in positive ion mode.
LC-MS operation control and the data acquisition process were accomplished using AB-Sciex Analyst processing software.
The B-vitamins separation was achieved on a Waters (Montplaisir, Netherland) Atlantis dC18 column (2.1 mm x 150 mm, 5 µm). The fat-soluble vitamins separation was achieved on an Interchim (Monluçon, France) Zorbax dC18 column (2.0 mm x 250 mm, 5 µm).
The liquid chromatographic system was equipped with a binary pump (Agilent 1100 pump), an Agilent 1100 autosampler and a Diode array multiple wavelength detectors (Agilent 1100).

2.3.2. Reagents

Thiamin hydrochloride, riboflavin, nicotinamide, pyridoxine hydrochloride, pyridoxal hydrochloride, nicotinic acid, pantothenic acid, α-tocopherol, γ-tocopherol, β-tocopherol, α-tocoacetate, lutein, β-sitosterol, ethanol absolute, acetonitrile, glyoxylic acid, hexane, sodium acetate, L-Glutathione reduced, Ethylene-diamine-tetra-acetic acid, Phosphatase acid from potato, taka-diastase from Aspergillus oryzae, beta-glucosidase were obtained from Sigma Aldrich (Saint Quentin Fallavier, France). Echinenone was purchased from CaroteNature (CaroteNature GmbH, Lupsingen, Switzerland). α-Tocotrienol, γ-tocotrienol and β-tocotrienol were obtained from CHEMOS (Chemos GmbH, Regenstauf, Germany). Pyrogallol was from Fluka. Methanol was from Biosolv. All chemicals were of analytical-reagent grade (purity>98%). Papain and Iron(II) Sulfate heptahydrate were purchased from Merck. Thiamin-d3-hydrochloride, pantothenic acid-13C3, 15N hemicalcium salt and nicotinamide-d4 were obtained from Toronto Reasearch Chemicals. For LC analysis, distilled water was further purified by passing it through a Milli-Q plus apparatus (Millipore, corporation France).

2.3.3. Determination of B-vitamins in the different food matrices

2.3.3.1. Sample hydrolysis and extraction

625 mg of sample were transferred into a 50-mL FALCON tube. 100 µL of mix internal standard solution (15.6 µg/mL) was then added. 4.75 mL of sodium acetate (pH 4.5) was added to the sample. 1.25 mL of 0.5M glyoxylic acid solution, 0.25 mL of 1 % L-glutathione reduced solution, 0.25 mL of 1% ethylene-diamine-tetra-acetic acid solution and 0.2 ml of 2% iron (II) sulfate heptahydrate solution were added to the sample. 1 mL of a mixture of enzyme solution in sodium acetate (pH 4.5) containing 2.5 mg of phosphatase, 12.5 mg of papain, 62.5 mg of takadiastase and 2.5 mg of β-glucosidase was added and, the mixture was strongly mixed and incubated at 37°C for 14h. The cooled sample was vortexed and centrifuged at 12,000 x g for 10 min. The supernatant was filtered through a 0.22 µm Millex filter (Milipore, MA, USA). 10 µL of the filtrate was injected in duplicate into the LC-MS/MS system

2.3.3.2. LC-(ESI)MS/MS analysis

Analytes were detected and quantified as in previous described method (Nurit et al., 2015).

2.3.4. Determination of lipid-soluble bioactive compounds in the different food matrices

2.3.4.1. Standard solutions preparation LC-(ESI)MS/MS analysis

Stock solutions of carotenoids and tocols isomers standards were stored at -20°C in ethanol. Using their respective coefficient of extinction, concentrations of standard solutions were determined by spectrophotometry after dilution in ethanol. An ethanolic working solution containing the six lipid-soluble compounds and the two internal standards (echinenone and α-tocoacetate were used as internal standards respectively for carotenoids and tocols quantifications) were daily prepared by dilution of the stock solutions. A calibration curve ranging from 7.8 µg/mL to 63 µg/mL was prepared by step-wise dilution of the mixed working solution with ethanol.

2.3.4.2. Sample extraction

Individual carotenoids, tocols derivatives and β-sitosterol were extracted as in a method previously described (Lyan et al., 2001). All extractions were performed at room temperature under yellow light, to minimize light-induced isomerization. Six hundred mg of flour, freeze-dried dough and bread or toasted bread were exactly weighted in a screw-capped tube, humidified with 1mL of dH2O and then deproteinized by addition of 1mL of ethanol containing the internal standards (echinenone and α-tocoacetate). Fat-soluble molecules were extracted twice by

the addition of 2mL of hexane containing pyrogallol. The mixture was mixed 30s and then centrifuged for 5 min at 500 g. Both upper hexane phases were collected and then evaporated to dryness under nitrogen. The residue was dissolved in 500 µL of acetonitrile/dichloromethane/MeOH mixture (70/20/10) and 200 µL were filtered through a 0.22 µm Millex filter (Milipore, MA, USA). The filtrated (10 µL) was injected in duplicate into the LC-MS/MS system.

2.3.4.3. Chromatographic conditions

LC separation was achieved using a solution of acetonitrile/dichloromethane/MeOH (70/20/10) as mobile phase. The flow rate was set at 0.4 mL/min.

2.3.4.4. Mass spectrometer conditions

The APCI interface was used for quantitative determination of the analytes. The MS conditions were optimized to obtain the highest sensitivity by direct infusion of standard solutions. Maximum sensitivity was obtained for a source temperature at 500°C. The nebulizing gas was adjusted to 50 psi and the heater gas to 60 psi. Each standard was optimized one by one to obtain the best sensitivity.

Quantification was achieved by selected ion monitoring (SIM) and by measuring product ions (multiple reaction monitoring [MRM]) from the fragmentation of the protonated [MH] + molecules. The collision energy potential and the collision exit potential were then adjusted to optimize the signal for the most abundant product (daughter) ions.

Concentration of each molecule in the sample was calculated using 6-point calibration curves ranging from 7.8 µg/mL to 63 µg/mL. Concentrations of echinenone, lutein, α-tocoacetate, α-tocopherol, β-γ-tocopherol, α-tocotrienol, β-γ-tocotrienol and β-sitosterol were calculated using a linear regression (concentration vs. peak area) of the six-point external standards curves and were adjusted by percent recovery of the added internal standard (Leenhardt et al., 2006).

The most important LC/APCI-MS parameters for SIM and MRM acquisition of the 8 target compounds are summarized in Table 2

Table2. LC/MS/MS parameters of the LC/PI/APCI/MS/MS method for the detection of fat-soluble molecules

Analyte	SIM[a] (m/z)	MRM[b] (m/z)	Lens parameters					
			Declustering Potential (V)	Focusing Potential (V)	Entrance Potential (V)	Cell Entrance Potential (V)	Collision Energy (V)	Cell Exit Potential (V)
α-tocopherol		431/165	11	346	6	69	33	0
β -γ-tocopherol	417		25	390	11	20	12	9
α-Tocotrienol		425/165	19	240	5	21	33	2
β -γ-tocotrienol	411		37	290	5	20	11	8
β-sitosterol		397/271	16	366	6	76	30	5
Lutein	551/175		9	400	7	25	27	2
α-tocoacetate		437/165	5	300	1.5	29	40	7.5
Echinenone		551/203	50	240	11	27	17.5	10

a : SIM: Selected ion monitoring

b : MRM: Multiple reaction monitoring

2.4. Statistical analysis

Duplicate samples of semi-coarse wheat flours produced by conventional and improved industrial milling and duplicate samples of milling fractions were taken for the determination of compound concentrations.

In order to test the influence of bread making steps on molecules content, two replicates of one bake were sampled and analyzed at selected steps (kneading, baking and toasting). The analysis of variance for the three different breadmaking steps highlighted significant differences ($p \leq 0.0001$) for all compounds (supplementary data 2).

Data were studied by variance analysis using the procedure GLM of Statgraphics Centurion XVI, Statpoint Technologies, Inc. Warrenton, Virginia, USA. Differences in the analyte contents were evaluated using a LSD test. Differences with $p < 0.05$ were considered significant.

Supplementary data 2 : ANOVA table F value computed for the three different breadmaking steps (df=2)

F	Breadmaking steps
Thiamin	89.85***
Nicotinic acid	7.70***
Nicotinamide	148.57***
Pyridoxale	94.01***
Pyridoxine	27***
Riboflavin	95.32***
Pantothenic acid	497.42***
α-tocopherol	62.22***
β -γ-tocopherol	29.13***
α-tocotrienol	16.25***
γ-tocotrienol	7.24***
β-sitosterol	9.39***
Lutein	58.57***

*** : $p \leq 0.0001$

Leenhardt, F., B., Rock, E., Boussard, A., Potus, J., Chanliaud, E., Remesy, C*. 2006. Wheat lipoxygenase activity induces greater loss of carotenoids than vitamin E during breadmaking. Journal of Agricultural and Food Chemistry 54, 1710-1715.*

Nurit, E., Lyan, B., Piquet, A., Branlard, G., Pujos-Guillot, *E. 2015. Development of a LC-MS/MS method for the simultaneous screening of seven water-soluble vitamins in processing semi-coarse wheat flour products. Analytical and bioanalytical chemistry DOI 10. 1007/ s00216-015-8553-1.*

3. Effects of environment and variety on B and E vitamin and Lutein, β-sitosterol contents in a worldwide bread wheat core collection and association study.

3.1. Plant material and field experiments

One hundred and ninety five accessions of bread wheat accessions (*Triticum aestivum L.*) were selected from the INRA worldwide bread wheat core collection of 372 accessions (Balfourier et al., 2007) to maximize the geographical diversity represented and minimize the risk of crop failure due to lodging. In addition, accessions originating from 70 different countries include both landraces from the 19th century and old or modern cultivars from the 20th century. All seeds were obtained from the INRA Clermont-Ferrand Genetic Resources Centre for Small Grain Cereals (www4.clermont.inra.fr/umr1095_eng/crg). The 195 accessions were grown in 2005-2006 at Clermont-Ferrand (CF), France and in 2006-2007 at Le Moulon (LM), France using regional farming methods which included applying appropriate fertilizers and pesticides. In CF, the weather conditions during the growing season were similar to the long-term average for this site, with conditions close to optimal. From sowing to harvest maturity, the crops received over 500mm of rainfall. However some periods, especially grain filling period were rather under heat- and water-stress conditions (Table 3). In LM, the cultivation took place in good conditions and except for a short period of mild water deficit during stem extension , the plant received a good supply of water (adding to over 600mm precipitation) (Plessis et al., 2013).

Table 3: **Precipitation (mm) and Temperatures maximal during the periods of 9 to 16 of June in Clermont-Ferrand for the year 2006**.

	Cultivation month and year	Harvesting Date (31 July 2006)
	June 2006	
Date	Rainfall	Maximum Temperatures (°C)
9	0	29
10	0	29.3
11	0	29.7
12	0	30.9
13	0	32.2
14	0	32.2
15	0	31.4
16	2.8	29.4

3.2. Traits measured

3.2.1. Grain traits

For each accession, grain was harvested in bulk and was used to estimate the different grain traits and the characteristics of the derived flour.

Thousand Kernel Weight (TKW), in grams was estimated by weighing in duplicate 250 kernels of each accessions and multiplying the total weight by 4.

3.2.2. Flour traits

Whole-grain flour was made using a Cyclotec mill (sieve 0.75 mm, mill 6800, FOSS electric A/S, Hilleroed, Denmark)(Bordes et al., 2008).

3.2.2.1. Near Infrared reflectance spectrometry (NIRS)

Visible –NIR spectra of the wholemeal flours were acquired using a NIRs system 6500 spectrometer (NIRs system, Inc., MD, USA). Reflectance spectra were taken from the 400nm to 2500nm region and recorded as log (1/R) at 2nm intervals. The WinISI II version 1.5 spectrum analysis software (InfraSoft International, Port Matilda, PS, USA) was used to operate the scanner and to develop analytical procedures. All spectra were collected over the 400-2500nm wavelength range and were used to assess grain protein content (GPC). GPC was estimated using NIRS and the AACC method 29-70A (AACC, 1995) with Kjeldahl calibration.

3.2.2.2. Analysis of Vitamins

B and E Vitamins as well Lutein and β-sitosterol were determined using the methods, which are specified above (2.3.3 and 2.3.4). All analysis were carried out in duplicate and corrected for moisture content.

3.3. Identification of mapping regions responsible for the variation in vitamin traits

We scanned the genome for gene or QTL involved in the genetic control of wheat vitamins composition by association mapping (AM). AM is a study of the relationship between phenotypic variation and molecular markers. The mapping of these markers allows the identification of the regions involved.

3.3.1. Genotypic data

Genotype data for the 195 accessions used were already available for 578 diversity array technology (DArT, Triticarte, Camberra, Australia; www.triticarte.com.au) (Bordes et al., 2011; Plessis et al., 2013). All the accessions were also genotyped with 282 SSR markers assigned to the 21 chromosomes and SSR data were obtained from previous studies on the core collection (Bordes et al., 2011; Plessis et al., 2013). Finally,

genotype data for the 195 accessions used were already available for 48 SNPs from previous study of Bordes et al. (2011) and for 149 SNPs markers from the study of Plessis et al.(2013)(Chapter 7-Supplementary data 3)

3.3.2. **Genetic map and Association analysis**

A consensus map using published data was built with the map described by (Bordes et al., 2011; Plessis et al., 2013) and was used as reference. For association analyses, rare alleles (<5 % of all alleles at a locus) were filtered as missing data in association analysis. Association between markers and vitamin traits were tested as by Bordes et al. (2011) using the general linear model (GLM) of the TASSEL version 3.0 software. GLM was performed using the population structure Q-matrix as covariates that give the contribution of a genotype to each of the five inferred ancestor groups (Yu et al., 2006) for the reduction of false positive. The significant markers-trait association was tested using an F-test. Association analysis was conducted on each of the two environments. Associations were considered as significant at $p \leq 0.01$. The variability of the traits explained by the marker as a percentage of the total variability was evaluated by the r^2 value. MapChart version 2.2 software (Voorrips , 2002) were used to represent consensus genetic maps markers tested and associated vitamin traits.

3.4. Statistical Analysis

Differences in the vitamin contents were evaluated using a LSD test. Differences with $p< 0.05$ were considered significant. To relate water or fat-soluble compounds values to other parameters of the kernels, Pearson correlation coefficients of individual water or fat-soluble compound contents with thousand kernel weights, protein contents and individual water or fat-soluble compound contents were calculated using Statgraphics Centurion XVI, Statpoint Technologies, Inc. Warrenton, Virginia, USA.

Analysis of variance (ANOVA) was carried out to determine genetic and environment variances among the traits measured in environments using the general linear model procedure of Statgraphics Centurion XVI.

The broad-sense heritability (H^2) was calculated as $H^2 = \sigma_g^2 / (\sigma_g^2 + \sigma_e^2)$, where σ_g^2 is the genetic variance and σ_e^2 is the environmental variance.

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Bordes, J., Ravel, C., Le Gouis, J., Lapierre, A., Charmet, G., Balfourier, F*. 2011. Use of a global wheat core collection for association analysis of flour and dough quality traits. Journal of cereal science 54, 137-147*

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Voorrips, RE*.2002. MapChart: Software for the graphical presentation of linkage maps and QTLs. The Journal of Heredity 93, 77-78.*

Yu, J., Pressoir, G., Briggs, WH., Vroh, Bi, I., Yamasaki, M., Doebley, JF., McMullen, MD., Gaut, BS., Nielsen, DM., Holland, JB., Kresovich, S., Buckler, E*S. 2006. A unified mixed-model method for association mapping that accounts for multiple levels of relatedness. Nature Genetics 38, 203-208.*

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Development of a LC-MS/MS method for the simultaneous screening of 7 water-soluble vitamins in processing semi-coarse wheat flour products

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1. Introduction

Nowadays there is an increasing demand from the consumers to get well-balanced foods with enhanced nutritional values, such as breads containing whole grain, multi-grain or other functional ingredients. Reincorporation of different fractions of wheat grains , like the bran or germ fractions which are normally removed during milling but known to contain most of the nutritive compounds may be useful to address this demand.

Among the micronutrients and phytochemicals contained in the bran and germ fractions, vitamins are an important group of bioactive compounds, which are required for the growth and the healthy maintenance of the human body.

Enrichment of vitamins in cereal food products is an important issue which has to be properly controlled to satisfy the nutrition of the consumers. As many vitamins are unstable and easily degraded, monitoring their loss during processing is important in the development of appropriate processing and storage schemes for optimal nutrient content in the final food products. Therefore, rapid and reliable analysis of vitamins is in high demand by wheat-based food manufacturers and wheat breeders.

Measurement of vitamins in foods is complicated and represents a complex analytical problem for several reasons. Firstly, because of the diverse chemical structures and properties of vitamins, it is very difficult to develop a single method for their simultaneous determination. Secondly, vitamins in wheat are present at relatively low concentrations that require sensitive methods for their analyses (Fardet, 2010). Finally the susceptibility of vitamins to degradation by exposure to light, air, heat, alkaline pH and their diverse occurring form make their extraction from food matrices very challenging.

The referenced analytical methods for the determination of water-soluble vitamins are mainly based on microbiological assays established for more than 25 years (Blake, 2007) with a large number of alternative methods (Ball, 2006).

However the weak precision and accuracy of the various microbiological assays have led to the development of well-validated liquid chromatography methods [Blake, 2007; Rizzolo and Polesello, 1992), but most of them are designed for a limited number of analytes, since analysis of water soluble vitamin requires specific chromatographic conditions.

Some methods use diode-array (DAD) and fluorescence detection systems for the analysis of pharmaceutical preparations and food supplements. However, detection at low wavelengths required for non-chromophoric vitamins like pantothenic acid is possibly subject to background interferences and noisy baseline (Trang, 2013). Fluorescence detection is a useful alternative for those vitamins that are naturally fluorescent (riboflavin and pyridoxine) or become fluorescent after derivatization (thiamin). Nevertheless, fluorimetric methods are ideals for pharmaceutical analysis, but can be limited when used for food analysis due to the

complexity of food matrices. Recently Langer and Lodge (2014) developed a method employing DAD and fluorimetric detection for the simultaneous quantification of six vitamins from a fortified food product. Unfortunately, this method cannot be used to determine the nutritional status of vitamins in wheat samples as it did not allow the quantification of pantothenic acid which is one of the major vitamins being present in wheat grains (Fardet, 2010).

Despite the potential of liquid chromatography/tandem mass spectrometry (LC-MS/MS), only a few methods have been applied for the determination of multivitamin in food samples [Gentili et al., 2008; Leporati et al., 2005; Baiyi et al., 2008). The extraction of the analytes from a matrix remains as the main limiting factor. The LC-MS/MS method developed by Leporati et al. (2005) allowed analyzing simultaneously thiamin, riboflavin, pyridoxal, pyridoxine, pyridoxamine, nicotinic acid and nicotinamide whereas folic acid and pantothenic acid needed an individual hydrolysis-extraction and were analyzed in separated LC runs. The procedure developed by Gentili et al. (2008) allows the analysis in a single chromatographic run of the only free forms of vitamins. In the objective of a large scale varietal screening, none of the above chromatographic procedures are suitable for the analysis of wheat flour and wheat food products.

The purpose of this work was to develop and validate a rapid but not absolute quantification LC-MS/MS method for the simultaneous determination of 7 water-soluble vitamins (thiamin, nicotinic acid, nicotinamide, pyridoxine, pyridoxal, riboflavin and pantothenic acid) in semi-coarse wheat flour as well as in dough, bread and toasted bread. Taking advantage of the high sensitivity and selectivity of the MS/MS detection, we have been able to considerably simplify the extraction procedure. The developed chromatographic method allowed the seven analytes mentioned above to be conveniently separated into three groups in less than 15 min. The combination of a rapid extraction, direct injection, shorter chromatographic separation and the use of only 3 internal standards has enabled us to achieve a sensitive high throughput quantification of several vitamins B in a very diverse range of food matrices. This approach is totally innovative and overcome the main difficulties reported in literature for the simultaneous vitamins analysis in complicated matrices such as wheat and wheat-based food products.

2. Results and discussion.

2.1. LC-MS/MS analysis

2.1.1. MS/MS

As the amounts of some vitamins in the different biological materials used in this study were rather low, multiple reaction monitoring (MRM), the most sensitive detection mode, was employed. Full scan and products scan under both positive and negative ionization modes were carried out for each vitamin by direct simple infusion. Regarding the negative ionization full scan mode, no relevant ions were detected in the mass spectra. Therefore we decide that the positive-ion was the suitable ionization technique for the detection of vitamins. The most abundant form of protonated molecules of thiamin, pyridoxine, pyridoxal, nicotinic acid, nicotinamide, riboflavin , pantothenic acid, thiamin-d3 , pantothenic acid-13C3,15N

and nicotinamide-d4 were [M+H]+(m/z=265), [M+H]+(m/z=170), [M+H]+(m/z=168), [M+H]+(m/z=124), [M+H]+(m/z=123), [M+H]+(m/z=377), [M+H]+(m/z=220), [M+H]+(m/z=268), [M+H]+(m/z=224), [M+H]+(m/z=127), respectively. These ten protonated molecular ion [M+H]+ were chosen as precursor ions in the subsequent MS/MS experiments.

Specific fragment-ions were obtained from these ten protonated molecules in the collision cell of the triple quadrupole. Table 1 (Chapter 3 "Material and methods"-paragraph 1.7) lists the characteristic ions and the corresponding collision energy for each vitamin. Collision-induced fragmentation (CID) led to fragment ions at m/z 122 for thiamin, at m/z 152 and 150 for pyridoxine and pyridoxal respectively, at m/z 243 for riboflavin, at m/z 90 for pantothenic acid. Loss of urea and carbon dioxide from nicotinamide and nicotinic acid produced in both cases a fragment ion at m/z 80. Decomposition of thiamin-d3 generated an abundant ion at m/z 125 Loss of water from pantothenic acid-13C3, 15N generated product ions at m/z 94. An intense fragment ion was observed for nicotinamide-d4 at m/z 84. Based on these precursors' ions and product ions, the MS/MS parameters were optimized as described in Material and methods.

2.1.2. LC column.

The aim of the study was to develop a rapid LC-MS/MS method suitable for simultaneous separation of all target analytes in a single run. Due to the chemical and structural diversity of water-soluble vitamins, it is difficult to find chromatographic conditions which allow the separation of the compounds of interest in a short run time with a good performance in terms of peak shape and peak intensity. However, recently, hydrophilic interaction liquid chromatography (HILIC) was proposed to improve chromatographic separation of hydrophilic vitamins (Gratacos-Cubrasi et al., 2011). Karatapanis et al. (2009) achieved the separation of six water soluble vitamins in 45 min using an end-capped HILIC-diol column. Nevertheless, the important timeframe for the chromatographic separation does not seems compatible with a high throughput method. Therefore we decided to evaluate the efficiency of different reverse column on the separation of the selected vitamins.

In preliminary tests, two C18 columns were chosen for their superior retention of polar compounds. The column A was a Waters Atlantis dC18 column (2.1 mm i.d. x 150mm, 5µm) and the column B was a Nucleodur Polar tech (Macherey-Nagel, EC 150/2,5um).

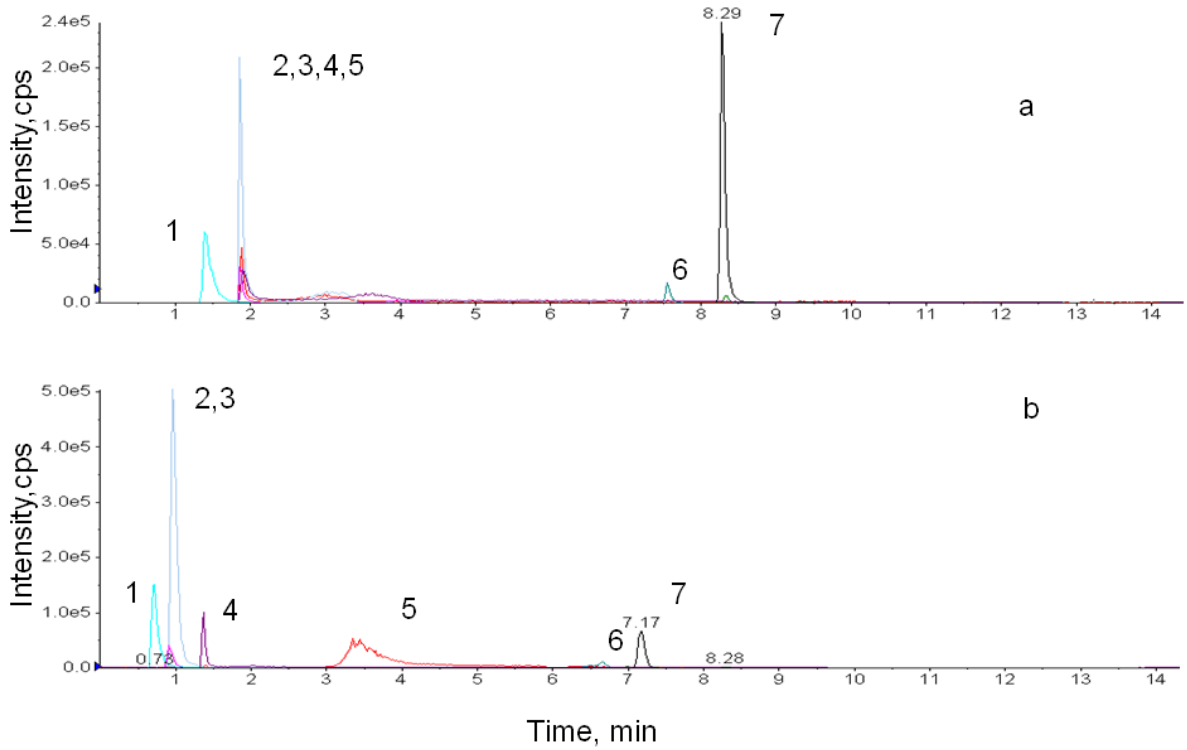
We decide to compare both columns in terms of separation and retention of pantothenic acid, thiamin, pyridoxine, pyridoxal, nicotinic acid, nicotinamide and riboflavin within the same chromatographic conditions. It was found (Figure. 1) that under the same chromatographic conditions as described in materials and methods, column A or B allow the separation of vitamins in 15 min. Column A did not separate

the pyridoxine, pyridoxal, nicotinic acid and nicotinamide, whereas column B led to a better separation of these four vitamins going with an improvement of the intensity of the well separated vitamins peaks (Figure. 1). The peak shape observed on the chromatogram for pantothenic acid and nicotinic acid after elution with column B was not satisfactory. It has also to be pointed out that the retention times observed for thiamin, pyridoxal and pyridoxine using column B were very short (0.70 and 0.90 min respectively) which could affect the sensitivity and reproducibility of the technique. In fact wheat flour hydrolysate exhibits a large amount of carbohydrates which were not separated and led to the fouling of the ion source of the mass spectrometer, thus resulting in a significant decrease of analyte responses.

In order to protect the MS from matrix contamination, the Valco Valve function was activated to split most of the LC effluent to waste during the first minute of the chromatographic method.

Nevertheless column A resulted in better performances in terms of peak shape for most of the vitamins and it allowed us to split most of the carbohydrates to waste as the first eluted vitamin was thiamin with a retention time of 1.39 min leading to a better long-term reproducibility. In addition, it has to be mention that even if four vitamins (pyridoxine, pyridoxal, nicotinic acid, nicotinamide) were chromatographically unresolved, the selectivity of a hyphenated technique such as LC/MS is enough to detect these vitamins in a single chromatographic run (Gentili et al., 2008).

Figure 1 Comparison between the LC-ESI-MRM chromatograms of a 2 µg/mL standard mixture.



(1) thiamin, (2) pyridoxine, (3) pyridoxal, (4) nicotinamide, (5) Nicotinc acid, (6) pantothenic acid, (7) riboflavin eluted from column (a) Atlantlis and (b) Nucleodur

2.2. Sample treatment

The goal was to find a procedure which allows the analysis of all the vitamins present in food products in a single chromatographic run and to simplify as much as possible the extraction procedure in order to apply this method at high throughputs.

The analytical procedure was optimized by taking into account both the nature of the analytes and the nature of the matrix. The determination of vitamins in food matrices is a very challenging analytical problem. First of all, vitamins are very complex micro-

constituents because of their diversity in structure and chemical properties. Vitamins are also very sensitive to pH, temperature, exposure to light, oxygen, and can be present in various forms in foodstuffs (free, bond to the proteins or polysaccharide as well as bond to phospholipids). From an analysis of the scientific literature (Ball, 2006) it appears that these vitamins are most often determined in the free form, involving hydrolysis of the phosphorylated form and those bound to proteins and polysaccharides. Different studies were done to determinate the optimal extraction procedure (Ndaw et al., 2000; Ndaw et al., 2002) (Table 2)

Table 2. Linear dynamic range (0.05-9µg/mL) of the detected water-soluble vitamins

Compound	n[b]	a x 10^{-5}[c]	r^2[d]
Thiamin	30	3.36	0.996
Nicotinic acid	25	3.10	1
Nicotinamide	30	1.85	0.991
Pyridoxal	25	4.52	0.992
Pyridoxine	25	2.76	0.999
Riboflavin	30	0.33	0.997
Pantothenic acid	35	0.48	0.997

a : Calibration slope

[b] Number of determination

[c] ± Values are confidence intervals for 99% probability level.

[d] Correlation coefficients

2.2.1. Enzymatic hydrolysis

The enzymatic mixture proposed by Ndaw et al. (2000) constitutes an excellent enzymatic system of reference for the analysis of the free and chemically bound form of vitamins in foodstuffs. By using the chromatographic conditions described in material and methods, we therefore tested the use of this enzymatic system on the different wheat food samples. In semi-coarse wheat flour, the enzymatic hydrolysis (Table 3) led to higher vitamin contents than without enzymatic hydrolysis, although the differences were not significant for thiamin, nicotinic acid and pantothenic acid. When enzymatic hydrolysis was performed on semi-coarse bread and on semi-coarse toasted bread, significantly higher contents were obtained for nicotinamide, riboflavin and thiamin, respectively. No modification of the other vitamins was in fact observed by applying the enzymatic treatment to semi-coarse bread and semi-coarse

toasted breads. Our results indicate that the bound vitamin forms are essentially present within the flour and that the extraction protocol used is adequate to analyze the total vitamin content whatever the nature of the foodstuff. Nevertheless, the extraction performed according to Ndaw et al al. (2000) is simple, fast, accurate and can be extended to different wheat food sample.

Table 3. Content of B-vitamins in semi-coarse wheat flour, semi-coarse bread and semi-coarse toasted bread obtained using the extraction with and without enzyme mixture.

Samples (µg/g of DM)	Thiamin	Nicotinic acid	Nicotinamide	Pyridoxal	Pyridoxine	Riboflavin	Pantothenic acid
Semi-coarse wheat flour							
Free vitamins	3.31±0.26	3.43±0.24	**0.55±0.06**	**0.19±0.02**	**0.59±0.04**	**0.18±0.05**	6.09±0.18
Enzymatic extraction	3.56±0.36	3.55±0.13	**0.71±0.07**	**0.29±0.03**	**0.78±0.06**	**0.37±0.05**	6.37±0.13
Whole wheat bread							
Free vitamins	1.50±0.14	5.76±0.39	**3.61±0.07**	0.37±0.11	1.36±0.24	**0.43±0.10**	1.77±0.4
Enzymatic extraction	1.83±0.06	5.57±0.09	**4.97±0.12**	0.63±0.08	1.91±0.39	**1.77±0.4**	2.86±0.2
Whole wheat toasted bread							
Free vitamins	**1.95±0.02**	8.24±0.34	7.01±0.65	0.67±0.01	3.54±0.35	0.93±0.1	2.12±0.11
Enzymatic extraction	**2.47±0.11**	7.29±0.00	7.65±1.08	0.52±0.16	3.30±0.39	1.19±0.05	3.01±0.37

Mean values ± S.D. for n=2 of the B-vitamins in food samples. Values highlighted in bold represent data significantly different (at 0.05% probability level) between enzymatic treatment for each vitamin in each food sample.

DM: Dry matter

2.2.2. Solid phase extraction

Different methods of solid phase extraction (SPE) were tested during the development of this procedure, but none of them gave satisfactory results. Because of the complexity of the matrices and the wide range of polarity occurring within the B-group vitamins, no SPE cartridges stationary phase was found to be specific enough. In addition, this sample preparation technique is really time consuming.

2.3. Matrix effect and the use of stable isotope-labeled internal standards

Due to the heterogeneous nature of the materials being studied, the important issue of matrix effect was investigated to avoid any interference which could induce bias during the analysis. Indeed molecules originating from the sample matrix that coelute with the compound(s) of interest can interfere with the ionization process in the mass spectrometer (Taylor, 2005), causing ionization suppression or enhancement.

2.3.1. Matrix effect

The post-extraction addition technique (Taylor, 2005; Matuszewski et al., 2003; Matuszewski, 2006) was used to determine matrix effects using the LC-MS/MS method.

“The post extraction addition technique requires sample extracts with the analyte of interest added postextraction compared with pure solutions prepared in the mobile phase containing equivalent amounts of the analyte of interest” (Taylor, 2005).

The results showing the matrix effects that may occur in different biological materials are presented in Table 4. Matrix effect determined for thiamin-d3-hydrochloride was respectively -71%, -79%, -78% and -82 % in wheat flour, dough, bread and toasted bread. These values represent a loss of 71 %, 79%, 78% and 82% of the thiamin-d3-hydrochloride signal in the respective material.

The ion suppression was lower in case of nicotinamide-d4 and no matrix effect was observed for pantothenic acid-13C3, 15N hemicalcium salt in dough and toasted bread. Thus, it appears clearly from these results that the more negative effect will concern the highly polar analytes such as thiamin (retention time 1.39min) and the polar analytes such as nicotinamide (1.94 min). These data confirm the presence of matrix effects and constrain us to consider the most appropriate way to remove matrix effect and ion suppression. In addition, we made the assumption that class of homologous compounds might be affected by an equivalent matrix effect. Three classes of homologous compounds were defined. The thiamin which is the most polar compounds was representative of the first class. Nicotinic acid, nicotinamide, pyridoxale and pyridoxine which are polar compounds were the second one. Finally, the low polar compounds, riboflavin and pantothenic acid were representative of the third class. In general, a stable isotope labeled-internal standard is considered to be adequate, since it shows an almost identical behavior than the analyte of interest in sample pretreatment, chromatography, and ionisation as well. Therefore, we

decided to use one isotope labeled internal standard by class of compounds. Thus, each labeled internal standard was used to correct for any variations (due to sample extraction and matrix effects) which could occur to any vitamins within a class.

Table 4. Calculation of matrix effects using the post-extraction spike method (by Matuszewski *et al. 2003*)

	Electrospray Ionization			
matrix effect (%)	wheat Flour	dough	bread	toasted bread
analyte ±Standard deviation				
thiamin-d3	-71 ± 1,11	-79± 1,65	-78 ±1,92	-82 ± 0,50
nicotinamide-d4	-27± 3,02	-36 ± 7,61	-13 ± 4,03	-12 ± 3,56
Pantothenic Acid-^{13}C3,^{15}N	+17 ± 2,04	+2 ± 0,68	+21 ± 6,40	+3 ± 2,04

(-% suppression; +%enhancement) of the observed matrix effects

2.4. Quantitative analysis

2.4.1. The use of stable isotope-labeled internal standards

We decided to use stable isotope labeled internal standard of Thiamin-d3 hydrochloride for thiamin. Nicotinamide-d4 for nicotinamide, nicotinic acid, pyridoxal and pyridoxine.Pantothenic acid- 13C3, 15N was used for pantothenic acid and riboflavin.

The excellent precision (Table 5) and bias (Supplementary data 1) of the method within the different materials validated the choice of using three internal standards

Table 5. Intra-day and Inter-day precision of the LC-MS/MS methods for the determination of water-soluble vitamins in selected food matrices

	Intra-day precision RSD (%)				Inter-day precision RSD (%)			
Compound	Wheat flour	Dough	Bread	Toasted bread	Wheat flour	Dough	Bread	Toasted bread
Thiamin	3.9	3.2	6.6	5.7	6.2	7.2	8.4	10.4
Nicotinic acid	5.9	2.9	2.2	4.3	7.2	7.1	8.8	6.1
Nicotinamide	7.9	6.5	5.1	4.4	10.7	8.1	7.9	7.6
Pyridoxal	4.9	9.1	5.7	6.3	11.0	9.7	9.4	9.8
Pyridoxine	5.0	4.1	6.8	9.3	10.4	10.8	9.5	8.9
Riboflavin	6.9	7.6	7.7	5.8	10.0	10.1	12.2	13.8
Pantothenic acid	4.9	4.4	3.5	5.8	5.1	6.9	9.9	9.9

RSD: Relative standard deviation

2.5. Method validation

2.5.1. Precision and bias

Intra-day and inter-day precisions shown in (Table 5) provide the evidence that short-term reproducibility was satisfactory for all the vitamins in every matrix (% RSD ranging from 2.2 to 9.3) and seemed to be lower than the long-term evaluations (%RSD ranging from 5.1 to13.8). It was demonstrated that for each vitamin in each food matrix, there were no significant difference between the added concentration and the recovered concentration (Supplementary data 1). Thus it confirms the bias of the developed method.

2.5.2. Linear range

As shown in Table 2 responses for all the vitamins were linear over the working range (0.05-9 µg/mL) with correlation coefficients close to one. Each regression was validated using an F-test.

2.5.3. Limit of quantification and analysis of food sample

From the results of table 6, it appears that the different LOQs combined with the wide linear dynamic range allowed us using this LC-MS/MS method to quantify the natural content of water soluble vitamins in foodstuff.

2.6. Application of the method to the analysis of selected food samples

The method was applied to the determination of water soluble vitamins in manufacturing wheat-based food products. Values obtained for the content of B vitamins at various breadmaking steps are shown in Table 6. The concentration of thiamin, pantothenic acid, pyridoxine and pyridoxal in semi-coarse wheat flour ranged from 4.25 to 0.97 µg/g DM. These values seem to be consistent with those previously reported (Fardet, 2010; Batifoulier et al., 2005). It can be noticed that, in general, the highest content is represented by nicotinic acid, which is the most stable compound among the various vitamins here considered.

The contribution of the kneading and fermentation is given by the content of vitamins in the dough. It has to be noticed that the dough exhibits a much higher content of riboflavin and nicotinamide than semi-coarse wheat flour. Pederson et al. (1989) showed that the fermentation step slightly increased the content of some B vitamins

The level of thiamin, pyridoxal, pyridoxine and pantothenic acid were found to be reduced from semi-coarse wheat flour to semi-coarse bread, in general the losses of those vitamins mostly due to the degradation of these molecules at the high temperatures applied during baking were similar to that observed earlier (Batifoulier et al., 2005, Pederson et al., 1989).

Table 6. Results of the determination of the vitamin content in selected food matrices

	Wheat Flour		Dough		Bread		Toasted bread	
Compound	(µg/g DM)	LOQ (µg/g)	(µg/g DM)	LOQ (µg/g)	(µg/g DM)	LOQ (µg/g)	(µg/g DM)	LOQ (µg/g)
Thiamin	4.25 (0.12)	0.9	4.27 (0.11)	0.3	2.54 (0.12)	0.3	2.47 (0.07)	1.08
Nicotinic acid	6.86 (0.35)	2.4	4.63 (0.17)	1.89	4.41 (0.17)	2.7	6.64 (0.45)	3.5
Nicotinamide	1.29 (0.08)	1	1.78 (0.05)	0.9	2.39 (0.01)	1.7	4.63 (0.14)	1.2
Pyridoxal	0.97 (0.05)	0.4	0.64 (0.01)	0.45	0.54 (0.02)	0.36	0.36 (0.02)	0.3
Pyridoxine	1.40 (0.08)	0.7	1.26 (0.05)	0.6	1.19 (0.05)	0.9	2.08 (0.09)	1.4
Riboflavin	0.44 (0.02)	0.27	0.65 (0.02)	0.27	0.69 (0.03)	0.2	0.76 (0.04)	0.09
Pantothenic acid	4.43 (0.16)	0.9	4.13 (0.12)	0.9	3.84 (0.14)	0.4	3.32 (0.08)	1.1

*Data are means of sixteen determinations (S.E.M in brackets).

LOQ: Limit of quantification

3. Conclusion

The developed LC-MS/MS method, allow a sensitive high throughput quantification of several water-soluble vitamins in a very diverse range of food matrices. Even if nicotinic acid, nicotinamide, pyridoxine and pyridoxal were not completely separated because of their structural analogies, the high sensitivity and selectivity of the MS/MS detection combined with the use of 3 internal standards, allowed the simultaneous determination of seven vitamins in a single chromatographic run. This approach is totally innovative and overcome the main difficulties reported in literature for the simultaneous analysis water-soluble vitamins in complex matrices such as wheat and wheat-based food products. The simplicity of the extraction procedure, as well as the direct injection of the extract in the LC-MS/MS system make our method rapid and potentially high-throughput. As a consequence, this procedure is suitable for a fast screening, but not absolute quantitative analysis of vitamin contents in wheat flour and wheat-based food products in an objective of a large scale varietal screening

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Supplementary data 1

Bias of water-soluble vitamins in selected food matrices with $t_{tabulated}$=3.355 (P=0.01, freedom degree 8)

		Wheat flour	Dough	Bread	Toasted bread
Analyte	Statistical parameter	Experimental value	Experimental value	Experimental value	Experimental value
Thiamin	t[a]	1.770	1.092	1.433	2.930
Thiamin	t'[b]	1.085	0.985	1.318	0.361
Nicotinic acid	t	2.409	3.221	0.692	0.249
Nicotinic acid	t'	1.293	3.162	0.247	1.207
Nicotinamide	t	0.760	0.886	0.204	0.559
Nicotinamide	t'	0.379	0.193	0.229	0.751
Pyridoxal	t	2.667	0.045	1.432	2.645
Pyridoxal	t'	1.815	2.190	1.284	0.181
Pyridoxine	t	2.254	1.274	2.258	0.501
Pyridoxine	t'	0.266	0.182	2.651	0.658
Riboflavin	t	2.169	0.109	3.201	2.233
Riboflavin	t'	0.165	0.124	1.576	1.846
Pantothenic acid	t	1.850	2.638	0.865	2.104
Pantothenic acid	t'	0.686	0.007	0.542	1.053

Bias parameter was validated using a spiked-recovery test. Ten determinations were obtained for each matrix and each analyte. The samples were spiked with the 7 vitamins at five concentration levels and each concentration was analyzed two times. Then both the original sample and the spiked ones were subjected to the sample preparation procedure and LC-MS-MS analysis. In order to determine if bias was acceptable the hypotheses that the slope was equal to one and the y-intercept was equal to 0 was tested. For both hypotheses a Student t-Test was performed.

If [a]t experiment $= \frac{|C1-1|}{Sc1}$ (which t distribution with n-2 degrees of freedom if the null hypothesis is true) was minor than $t_{tabulated}$ (P=0.01, freedom degree 8), it was not significantly different from 1

If [b]t'experiment = $\frac{|Co|}{Sco}$ (which t distribution with n-2 degrees of freedom if the null hypothesis is true) was minor than $t_{tabulated}$ (P=0.01, freedom degree 8), it was not significantly different from 0

When the two hypotheses were verified it was concluded that the bias of the method was acceptable.

Change in B and E vitamin and Lutein, β-sitosterol contents in industrial milling fractions and during toasted bread production

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1. Introduction.

Humans require 49 nutrients to meet their metabolic needs (Welch and Graham, 2003). Among them, the vitamins, which are grouped as micronutrients, are usually supplied within a well-balanced food intake in developed countries. One of the major food intakes is given by cereals and cereal-based food such as breakfast cereals, bread, cookies, extruded snack, etc. Lately, the consumer's interest is to get more nutritious wheat-based food products, as a lot of epidemiological studies have clearly shown the health benefits of consuming whole - grain cereals products (Fardet, 2010). Wheat (*Triticum aestivum*) is by far the most important crop for breadmaking because of its higher baking performance in comparison with all other cereals. A lot of scientific studies have been conducted to evaluate the impact of processing for manufacturing attractive wheat grain products. The most available literature reveals that the levels of micronutrients such as B and E vitamins in a finished product depend upon the food processing operations carried out. For example milling (Batifoulier et al., 2006, 2005; Doblado-Maldonado et al., 2012) stage have a detrimental impact on the tocol and on the B vitamin contents. In fact, the degree of refining is probably the most important factor (Batifoulier et al., 2005). Indeed, most of the B vitamins are present in the bran and germ: therefore, milling or extensive extraction is liable to severely reduce B vitamins in the flour (Doblado-Maldonado et al., 2012). Nevertheless, new milling technologies have been developped to produce flours with enhanced nutritional values. Conventional milling of grains was used to dissociate and separate the endosperm from the outer layers with the highest possible yield and purity (Hemery et al., 2007). Since most of the bioactive compounds are in the bran and especially in the aleurone layer (Antoine et al., 2002) different processes to separate wheat aleurone into purified fraction have been investigated (Hemery et al., 2007, 2009). Thus, new wheat mill fractions could be used as a functional ingredient in order to enrich wheat-based food products in bioactive compounds. It appears from the literature that baking stage may also severely depress the content of vitamin levels in products made from wheat (Batifoulier et al., 2005; Leenhardt et al., 2006). As reported by Watanabe and Ciacco (1990), the cooking water caused greater losses of thiamin, riboflavin and niacin in spaghetti than drying treatments. More recently, Hidalgo et al., (2010a) demonstrated that during the production of bread, the loss of carotenoids was different throughout the processing stages. Kneading led to limited degradation, bread leavening has almost negligible effects while baking has a marked influenced on carotenoids loss in bread. The stability of B-vitamins during the course of bread-making processes was investigated by Batifoulier et al. (2005). They observed that whole bread made with yeast resulted in 30% enrichment in riboflavin if the fermentation was sufficiently long and that pyridoxine content was lower in dough after kneading than in flour. Currently, knowledge

about the impact of breadmaking on specific bioactive compounds is plethoric, while no information is available about the effect of toasting on tocols, Lutein, β-sitosterol and water-soluble vitamins composition of toasted bread.

Therefore, the objective of this study was first to determine by means of LC-MS/MS and stable isotope dilution assay (Nurit et al., 2015), the B and E vitamins, Lutein and β-sitosterol in different industrial milled fractions to evaluate which of these show the highest health benefits and secondly to compare the variability of these compounds contents at each step of the breadmaking process of toasted bread produced with semi-coarse wheat flours (French flour, type 110) obtained by flour milling industry.

The effect of breadmaking and toasting on the concentration of these compounds in toasted bread made from the enriched flour was particularly discussed.

2. Results and Discussion

2.1. Bioactive compounds composition of milled wheat fractions

2.1.1. Contrasted B vitamins composition in the different milling fractions

The mean of B vitamin content in the different milling fractions is shown in Table 2. As expected the lowest vitamin contents were found in the white flour fraction, reflecting a poor accumulation of these vitamins in the starchy endosperm fraction. The concentrations of nicotinic acid, pyridoxal, pyridoxine and pantothenic acid were significantly higher in the coarse bran than in the other bran fraction while the concentration of thiamin (20.87 µg/g DM) was the highest in the enriched fraction. The present values were consistent with those previously reported within the same fraction for thiamin (Antoine et al., 2002) but with some differences for nicotinic acid, nicotinamide, pyridoxine and pyridoxal. Indeed, the content of the vitamins in our study were respectively 84 % and 64 % lower than the contents reported by Antoine et al., (2002). The variability in these vitamins was accounted for by the wheat varieties composing the industrial flour used, by environmental growing conditions probably influencing the metabolites in the sample analysed, and also by methodology used. As mentioned in a previous study (Nurit et al., 2015) the extraction was not carried out in alkali or strong acid as recommended in the standard methods. The enzymatic preparation used in our study may not have allowed the complete release of the vitamins present in the sample as nicotinamide adenine dinucleotide, nicotinamide adenine dinucleotide phosphate, nicotinic acid glucoside and pyridoxine glucoside. Glycosylated forms of pyridoxine and nicotinic acid are prevalent in plant-derived foods and contribute to human nutrition as partially available sources of pyridoxine and nicotinic acid (Gregory et al., 1991). Further investigations would be necessary to better understand the distribution of bound vitamins within the different fractions. No significant difference was observed for nicotinamide concentration in the milling fractions. Interestingly, important amount of B vitamins were recovered within the discard sifting

Table 2. B vitamin levels in milling fractions of wheat

Milled fractions	Thiamin	Nicotinic acid	Nicotinamide	Pyridoxine	Pyridoxal	Riboflavin	Panthotenic acid	Total
	µg/g DM							
White Flour	2.70[d]	13[d]	1.2[b]	1.3[c]	0.77[d]	0.83[c]	3.61[d]	23.41[d]
Coarse Bran	8.88[c]	41.66[a]	6.96[a]	11.36[a]	4[a]	3.96[b]	32.28[a]	109.09[a]
Thin Bran	9.36[c]	28.4[c]	5.11[a]	8.43[b]	2.39[c]	3.57[b]	26.17[b]	83.43[c]
White midds	16.97[b]	30.83[b,c]	5.55[a]	7.45[b]	2.59[b,c]	3.75[b]	17.37[c]	84.50[c]
Brown midds	18.31[b]	32.51[b]	6.3[a]	8.93[b]	3.2[b]	5.1[a]	20.08[c]	94.43[b]
Discard sifting	15.87[b]	32.23[b]	5.01[a]	7.79[b]	2.26[c]	3.58[b]	19.15[c]	85.89[c]
Enriched	20.87[a]	32.92[b]	6.34[a]	8.29[b]	3.33[b]	4.53[a,b]	16.84[c]	94.11[b]

Mean values for n=2 of the B vitamins in milling fractions. Different superscript letters within a column indicated statistically differences at the probability level < 0.05.

DM dry matter

2.1.2. A strong accumulation of E vitamins, lutein and β-sitosterol was reported in the enriched fraction

The concentration of tocols, lutein and β-sitosterol in the different milling fractions is given in Table 3. The concentration of total tocopherol in semi-coarse wheat flours determined in our work (Table 4) was 40% lower than the value for total tocopherol in whole flour from bread wheat reported by Ward et al., (2008) and Shewry et al., (2012). The effect can be explained by the fact that our extraction procedure did not include a saponification step which has been shown to provide significantly higher tocol recoveries (Panfili et al., 2003). β-sitosterol, lutein, α-tocotrienol and α-tocopherol concentrations were significantly ($p<0.05$) higher in the enriched fraction than in any other milling fractions, with respectively 413 µg/g, 7.05 µg/g, 10.57 µg/g and 16.09 µg/g DM. Even if several reports demonstrated that α-tocopherol was

being mainly concentrated in the germ (Hidalgo et al., 2007; Ko et al., 2003), the high level of α-tocopherol (which has been labelled as the most efficient antioxidant for breaking free-radical driven chain in-vivo) reported in the enriched fraction is of great importance as the objective is to reincorporate this fraction in white wheat flour and produce safe and healthy whole wheat food products. Significant quantities of tocotrienol in the bran were reported by Hidalgo et al. (2007) and in the hull and red dog fraction by Ko et al., (2003) which was in accordance with the high contents of α-tocotrienol recovered in the enriched fraction in our study. There was a major contribution of β-γ-tocotrienol (29.53 µg/g DM) to the total tocol content in the endosperm fraction. A similar trend was observed in millstreams fraction by Hidalgo et al. (2007) in bread wheat. The highest concentrations of lutein observed in the enriched fraction (7.05 µg/g DM) and white flour (4.67 µg/g DM) confirmed that lutein is mainly present in the starchy endosperm of the grain. It was therefore unfortunate that the β-γ-tocopherol, which was the tocol with the highest content, was mainly located in the coarse bran fraction (87.13 µg/g DM). The results showed that the enriched fraction as a functional ingredient could be used to enrich refined flour in these bioactive compounds, leading to a nutritional improvement of wheat flour. In addition, the improved industrial milling process should be further investigated in order to describe within the milling process the step responsible for the strong accumulation of vitamins B and of lipid-soluble bioactive compounds in the discard sifting fraction and also the fluorescence of the enriched fraction should be exploited to detect and quantify the proportions of the different grain tissues (aleurone, pericarp or germ) that compose the technological enriched fraction.

Table 3. Tocols, Lutein and β-sitosterol levels in milling fractions of wheat

Milled fractions	α-tocopherol	β -γ-tocopherol	α -tocotrienol	β- γ -tocotrienol	lutein	β-sitosterol	Total
	µg/g DM						
White Flour	3.42^{d}	4.44^{f}	2.34^{d}	29.53^{b,c}	4.67^{b}	55.6^{d}	100.01^{d}
Coarse Bran	0.76^{d}	87.13 ±^{a}	6.37^{c}	30.47^{b,c}	1.99^{c}	142.81^{c}	269.53^{c}
Thin Bran	2.48^{d}	56.87^{c}	7.82^{b,c}	41.24^{a,b}	4.19^{b}	126.8^{c}	239.40^{c}
White midds	8.25^{c}	29.93^{e}	5.3^{c}	28.79^{b,c}	3.01^{b,c}	220.98^{b}	296.27^{c}
Brown midds	9.66^{c}	83.59^{a}	5.9^{c}	29.23^{b,c}	3.52^{b}	281.92^{b}	413.82^{b}
Discard sifting	12.44^{b}	41.08^{d}	8.96^{a,b}	42.88^{a}	3.68^{b}	274.89^{b}	383.93^{b}
Enriched	16.09^{a}	72.6^{b}	10.57^{a}	38.05^{a,b,c}	7.05^{a}	413.27^{a}	557.62^{a}

Mean values ± S.D for n=2 of the liposoluble compounds in milling fractions. Different superscript letters within a column indicated statistically differences at the probability level < 0.05.

DM dry matter

Table 4. Results of the determination of the vitamin, lutein and β-sitosterol content in selected food matrice

Compounds	Wheat flour (µg/g DM)	Dough (µg/g DM)	Bread (µg/g DM)	Toasted bread (µg/g DM)
Water-soluble				
Thiamin	4.25 (0.12)	4.27 (0.11)	2.54 (0.12)	2.47 (0.03)
Nicotinic acid	6.86 (0.35)	4.63 (0.17)	4.41 (0.17)	6.64 (0.45)
Nicotinamide	1.29 (0.08)	1.78 (0.05)	2.39 (0.01)	4.63 (0.14)
Pyridoxal	0.97 (0.05)	0.64 (0.01)	0.54 (0.02)	0.36 (0.02)
Pyridoxine	1.40 (0.08)	1.26 (0.05)	1.19 (0.05)	2.08 (0.09)
Riboflavin	0.44 (0.02)	0.65 (0.02)	0.69 (0.03)	0.76 (0.04)
Pantothenic acid	4.43 (0.16)	4.13 (0.12)	3.84 (0.14)	3.32 (0.08)
Fat-soluble				
α-Tocopherol	5.02 (0.26)	1.47 (0.16)	1.49 (0.15)	3.57 (0.14)
β -γ-Tocopherol	9.38 (0.38)	6.21 (0.30)	5.10 (0.39)	7.09 (0.27)
α-Tocotrienol	1.03 (0.03)	1.19 (0.14)	1.26 (0.14)	1.83 (0.06)
β-γ-Tocotrienol	12.78 (0.48)	10.28 (0.85)	9.78 (0.50)	12.33 (0.68)
β-Sitosterol	111.17 (3.42)	80.40 (3.39)	73.98 (3.54)	84.79 (3.88)
Lutein	0.91 (0.05)	0.29 (0.07)	0.53 (0.03)	0.26 (0.02)

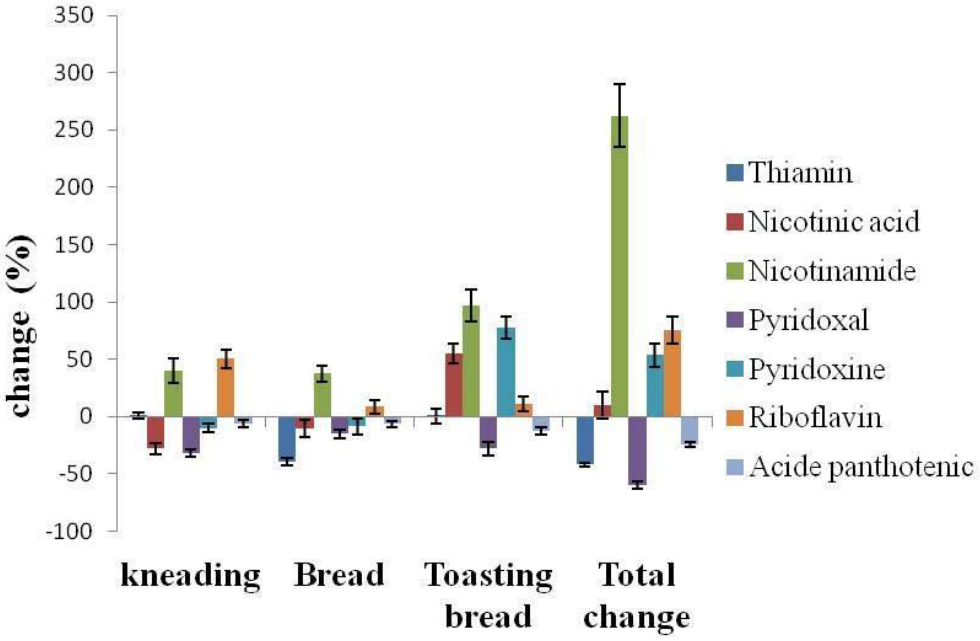
Data are means for 16 replicates (S.E.M in brackets)

2.2. Effect of breadmaking and toasting on bioactive compounds content

2.2.1. A significant decrease of thiamin, pantothenic acid, pyridoxine and pyridoxal contents occurred during bread making

In our study, the bread making caused significant evolution of B vitamins (Figure 1). Indeed, bread making elicited significant losses of thiamin (-39%), pyridoxal (-42%), pyridoxine (-12%) and pantothenic acid (-12%), compared to the original flour. This observation was in general agreement with previous work (Tanphaichitr, 2001) which reported a loss of 5-35% thiamin during breadmaking. The kneading step led to a severe loss of pyridoxal (-32%) whereas the baking step resulted in a more moderate (-15%) depletion of the vitamin.The decrease during these two stages was less drastic for pyridoxine (-10% and -9% respectively), which confirmed that pyridoxal was easily destroyed by heat and oxygen as mentioned by Batifoulier et al. (2005).

Figure 1. Effect of the breadmaking and toasting process expressed in percent (-% looses; +%enhancement) from the previous step on B vitamins



Data are means for 16 replicates ± SEM

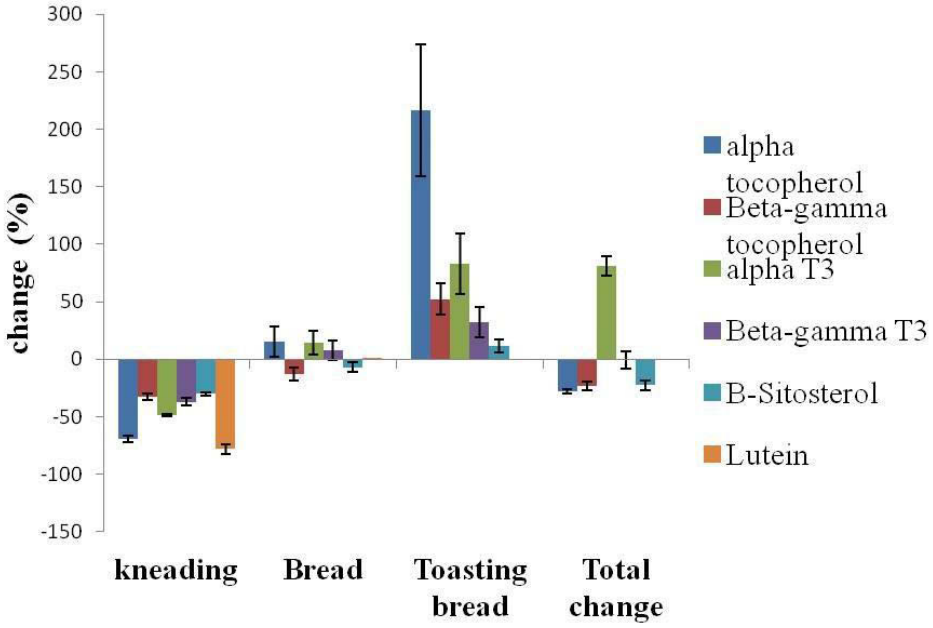
2.2.2. Contrasted variations for nicotinic acid, nicotinamide and riboflavin

There was a striking increase in riboflavin and nicotinamide content (+50% and +40% respectively) during kneading stage although this increase was maintained during the baking stage for nicotinamide (Figure 1), meanwhile the concentration of nicotinic acid during kneading stage decreased by a considerable extent (-28%). According to Batifoulier et al. (2005), upon yeast addition, dough generally exhibited a much higher content of riboflavin. Thus, as suggested by Leblanc et al. (2013) it could be expected that the food industry will exploit novel and efficient vitamin-producing strains to produce fermented products with elevated levels of these essential compounds. While to the best of our knowledge no data are available in the literature about nicotinamide content during wheat breadmaking, Mihhalevski et al. (2013) reported an increase in the concentration of nicotinamide during fermentation with lactic acid of the rye dough

2.2.3. A significant decrease of lipid-soluble bioactive compound contents occurred during kneading phase

α-tocopherol, β-γ-tocopherol, α-tocotrienol, β-γ-tocotrienol, β-sitosterol and lutein degradations during the kneading phase were relevant with average losses of 69%, 33%, 49% , 37%, 30% and 78% respectively (Figure 2). Our data are similar to the results reported by Hidalgo et al., (2010b) who observed average losses over all flours of 21.4% during the kneading phase of bread. As mentioned by Hidalgo et al. (2010a) and Leenhardt et al., (2006), the high degradation rates of lutein during the kneading phase were probably related to direct oxidation as well as the presence in wheat flour of different enzymes such as lipoxygenase and peroxidase. Irrelevant losses of tocols during bread baking observed in our data were likewise observed by Hidalgo et al., (2010b). The analysis of the breadmaking processes suggested that the kneading step has a more prejudicial impact on tocols content than the baking step. Therefore, as suggested by Leenhardt et al., (2006) a reduction of the kneading time and intensity associated with a longer period of dough fermentation may maintain a highest content of tocols in the whole wheat bread.

Figure 2. Effect of the breadmaking and toasting process expressed in percent (-% looses; +%enhancement) from the previous step on fat-soluble compounds



Data are means for 16 replicates ± SEM

2.2.4. Strong toasting impact on some B and E vitamins

Toasting led to an important loss (-13%) of pantothenic acid whereas baking resulted in a more moderate (-6%) depletion. There was no previous information on the impact of baking on pantothenic acid levels. Thus, heat had a negative but moderate impact on this vitamin during baking and a more detrimental impact during toasting. As shown by Adrian (1971), the humidity seemed to protect pantothenic acid when the temperature was low (100°C) and to accelerate its destruction when the temperature was higher.

The toasting phase induced a significant ($p< 0.0001$) increase of α-tocopherol (+216%), of β-γ-tocopherol (+52%), of α-tocotrienol (+83%), of β-γ-tocotrienol (+32%), of nicotinic acid (+55%), of nicotinamide (+97%) and of pyridoxine (+77%). This effect cannot be explained by the drying process. A more plausible explanation for higher content of these vitamins may be related to the extraction efficiency dependant of the food matrix before analysis. As explained above in 3.1.1 and 3.1.2, the extraction procedure used for the analysis of the different food matrix did not allow the release of all bounding forms present within the different matrix. Moreau et al. (1999) and Carpenter et al. (1988) reported that method including alkaline processing and heat treatment may have released vitamins from the compounds with which it was associated. It appeared that toasting treatment liberated bound tocols, nicotinic acid, nicotinamide and pyridoxine from their chemical linkage. These experiments have provided evidence that heat treatment (21 min at 200°C) of

semi-coarse wheat bread enhanced dramatically the levels of B and fat soluble vitamins and made them nutritionally available in toasted bread. From an industrial perspective, the effect of kneading and baking on bound vitamins should be further investigated in order to describe the stability of such compounds during breadmaking**.**

3. Conclusion

The literature about development of new technological processes to improve the nutritional quality of cereal products is plethoric. However this study is the first to report the impact of the toasting process on B and E vitamin and Lutein, β-sitosterol contents. Our results demonstrated that the toasting process can release bound bioactive compounds and led to enhance the nutritional quality of toasting bread. The enriched fraction obtained with an industrial milling process showed promising results in the objective of being used as a functional ingredient in order to enrich wheat-based products in lipid-soluble bioactive compounds. The niacin in mature cereal grains is present largely as chemically bound forms of nicotinic acid that are nutritionally unavailable. Methods which measure the total niacin (i.e., free plus bound) content of the food sample may be providing a gross overestimate of the biologically available niacin. In order to understand how processing can impact the level of bioactive compounds and modify their bioavailability, more research have to be undertaken to clearly define in the mature wheat kernel the repartition of the main bound vitamins within the different tissues. It would be interesting also in a near future to investigate the impact of breadmaking on the bound form of vitamins, and to determine the stability of the combined form of vitamins under kneading and baking processes.

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Effects of environment and variety on B and E vitamin and Lutein, β-sitosterol contents and quality traits in 195 bread wheats in a worldwide core collection

1. Introduction

Wheat is an important source of proteins, minerals, carbohydrates and vitamins which are molecules required for the growth and the healthy maintenance of human body. Thirteen vitamins are universally recognized at present, and they are classified into two groups according to their solubility. The fat-soluble vitamins are represented by vitamins A, D, E and K, and are soluble in fat. The water-soluble vitamins include vitamin C and the members of the B-group, namely B1 (thiamin), (B2) riboflavin, B3 (nicotinic acid + nicotinamide), vitamin B5 (pantothenic acid), B6 (pyridoxine, pyridoxal, pyridoxamine), B8 (biotine), B9 (folate) and B12 (cobalamin). Nowadays, it is becoming evident that many of the health benefits associated with the consumption of whole grain cereal products relate to the enhanced intake of micronutrients, phytochemicals and dietary fibers (Fardet, 2010). However, it has been suggested that intensive wheat breeding programs may have resulted in decreased content of bioactive components, by focusing on yield and processing quality (Shewry et al., 2011a). Lately, a number of comparative studies have investigated the nutrient composition of B vitamins in wheat lines. Davis et al. (1981) evaluated thiamin, riboflavin, niacin and pyridoxine content in wholemeal samples of 231 varieties grown on 49 locations over three years (406 samples in total). The distribution of thiamin, riboflavin and pyridoxine in flours and reconstituted wholemeals of 49 cultivars has been reviewed by Batifoulier et al. (2006). In 22 American and Canadian wheat lines, Sampson et al. (1996) have conducted the evaluation of pyridoxine and pyridoxine glucoside contents. Studies of Batifoulier et al. (2006) and Sampson et al. (1996) indicated that the content of vitamin B1, B2 and B6 varies considerably between cultivars. The availability of data from Davis et al. (1981) for the different classes of wheat grown in different environments (ie sites or years), has allowed them to study the variation in composition of thiamin, riboflavin, niacin and pyridoxine according to the environment and wheat classes. It appears from their study that thiamin and riboflavin content were significantly different by class and growing location. Whereas pyridoxine varied significantly by year and by class and niacin content were significant only by year. The HEALTHGRAIN diversity screen (2005-2010) has identified sources of variation in grain composition using a two stage strategy. One hundred fifty wheat lines were grown on a single site in 2005. Those lines were initially selected to study the extent of genetic variability for tocols, sterols, alkylresorcinols, phenolic acids, folate and

fiber components (Lampi et al., 2008; Andersson et al., 2010). These first analyses have allowed the selection of a smaller sub-set of 26 lines which were grown on the same site for two further years (2006, 2007) and on three additional sites (UK, France and Poland) in 2007 only. During second stage, these lines were subjected to the same range of analyses as in the initial diversity screen, and four other B vitamins (bioavailable niacin, thiamin, riboflavin and vitamin B6) were added (Shewry et al., 2011b). The HEALTHGRAIN diversity screen has provided evidence that variation existed in the contents of bioactive components and that the heritability in amount of bioactive components varied. Being high for tocols, sterols alkylresorcinols and arabinoxylan (about 50%) but rather low for folate, phenolic acids, thiamin, riboflavin, bioavailable niacin and vitamin B6 (below 30%) (Shewry et al., 2012). Furthermore, these components were not related to the age and origin of the lines, or to the agronomic and functional properties (Shewry et al., 2012). In addition, they identified wheat lines in which high levels of one or more groups of bioactive components were combined with acceptable yield and processing quality (Ward et al., 2008). However, because no robust and high throughput analytical methodologies were available, most of the reported studies have only described the effect of genotype and environment on limited class of compounds (Davis et al., 1981) or on limited lines (The HEALTHGRAIN study (2005-2010). Therefore the objectives of the present study were to describe the variability in the content of 6 fat and 7 water soluble vitamins of a large international bread wheat core collection grown in two contrasting growing location (Clermont-Ferrand, France and Le Moulon, France). A core collection of 195 accessions has been chosen from the INRA worldwide bread wheat core collection of 372 accessions which were based on passport and SSR marker data from the whole set of 10,000 accessions curated by the Clermont Ferrand genetic resources Center (Balfourier et al., 2007). This core collection is representative of the world's wheat diversity and includes almost all French wheat varieties, ranging from those derived from landraces used during the 19th century to the more recent varieties, as well as accessions from about 70 other countries (Bordes et al., 2008). The present study describes the genetic and environmental factors affecting the total contents of thiamin, riboflavin, pantothenic acid, the bioavailable forms of vitamin B6 and niacin (B3) and the free forms of fat-vitamins, using the method developed in the first paper (chapter 4), as well as the main grain traits determining wheat quality in this core collection. In addition, the correlation between kernel characteristics and the B and Fat-vitamin contents has also been determined and discussed.

2. Results and discussion

2.1. Effect s of genotype and environment on B vitamin contents in the bread wheat core collection

2.1.1. Variation in B vitamins of 195 bread wheat genotypes grown at two locations

The mean content and range of variation for the thirteen analysed vitamins are shown in table 1.The results indicate an important variability in B vitamin concentrations for the analyzed wheat cultivars. The thiamin concentration averaged 4.77 µg/g dry matter (DM) for the 195 accessions, ranging from 2.67 to 7.78 µg/g DM. There were 15.6, 17.7 and 10.83-fold differences between maximum and minimum values for nicotinamide, riboflavin and pantothenic acid contents, respectively. The mean nicotinic acid concentration was 2.76 µg/g DM, ranging from 1.31 to 6.22 µg/g DM. The pyridoxal concentration ranged from 0.21 to 1.90 µg/g whereas the pyridoxine concentration averaged 1.08 µg/g DM , ranging from 0.4 to 2.58 µg/g DM. Shewry et al. (2011b), recently reviewed the contents of B vitamins in whole wheat flour. The ranges were 5.53-13.55 µg/g DM of thiamin, 0.77-1.40 µg/g DM of riboflavin, 0.16-1.74 µg/g DM of niacin and 1.27-2.97 µg/g DM of vitamin B6. The contents of thiamin, riboflavin and vitamin B6 determined in the current study are therefore in line with this previous report. However, the contents of bioavailable niacin were higher than reported by Shewry et al. (2011b). In both studies, only the available form was determined. Though, the variation observed by Shewry et al. (2011b) was not as wide as in the present study, probably due to the small number of genotypes analyzed. Based on analyses reported by Davis et al. (1981) which ranged the content of vitamin B6 in whole meal samples of 231 varieties grown on 49 locations over three years from 1.6 to 7.9 µg/g with an overall mean of 4.6 µg/g, the values reported in our study were substantially lower. However, as mentioned in a previous study (Nurit et al., 2015), glycosylated forms of pyridoxine (human exhibited approximately 50% bioavailability for pyridoxine glucoside (Gregory et al., 1991)) were not determined in the present study. Thus it could explain the differences observed. Interestingly, the contents of nutritionally available form of vitamin B6 reported in this study are within the same range than total vitamin B6 content reported by Shewry et al. (2011b). It can be concluded that the contents of biologically available vitamin B6 are quite high in the bread wheat core collection. As reported by Sampson et al. (1996), vitamin B6 concentration varies significantly within wheats, and that pyridoxine glucoside accounts for an important percentage (average of 68 %) of vitamin B6 in wheats. When the data of all environments were considered, there were statistically significant differences in the B vitamin contents among the genotypes (Table 2), due to a pure genotype effect.

Table 1. Average B and E vitamins and Lutein, β-sitosterol content (Micrograms per Gram of Dry Matter) of the 195 wheat accessions grown at two locations.

Components	Mean contents	Range of contents	Heritability
Water-soluble Vitamins			
Thiamin	4.77	2.67-7.78	0.872
Nicotinic acid	2.76	1.31-6.22	0.78
Nicotinamide	0.88	0.23-3.59	0.65
Pyridoxal	0.74	0.21-1.90	0.65
Pyridoxine	1.08	0.4-2.58	0.69
Riboflavin	0.85	0.26-4.62	0.8
Pantothenic acid	5.92	1.89-20.48	0.89
Tocols			
α-Tocopherol	3.08	0.41-6.39	0.85
β -γ-Tocopherol	15.77	3.09-35.94	0.78
α-Tocotrienol	1.04	0.09-2.94	0.87
β- γ- Tocotrienol	10.69	3.38-23.59	0.88
Sterols			
β-Sitosterol	63.03	4.58-227.97	0.92
Carotenoids			
Luteins	0.41	0.02-1.77	0.97

Table 2. Sum of squares of variance analysis for B vitamins.

Source of variation	Degree of freedom	Thiamin	Riboflavin	Nicotinic acid	Nicotinamide
Genotype	195	286.82*	88.86*	210.40*	30.22*
Location	1	81.52*	0.64*	190.53*	125.08*
Error	580	109.13	51.04	136.78	31.18
		Pyridoxal	Pyridoxine	Pantothenic acid	
Genotype	195	16.05*	34.40*	1219.35*	
Location	1	7.54*	0.16*	3404.18*	
Error	580	16.71	31.44	397.81	

*P value <0.001.

2.1.2. Variation of B vitamin contents among growing locations (Le Moulon and Clermont-Ferrand)

Contrasted variation was observed in the B vitamin contents of 195 wheat genotypes grown at two locations. The mean values for all lines per site are given in Table 3. The level of vitamins was significantly different in each location as measured with the LSD test ($p<0.05$). The average thiamin, nicotinic acid, nicotinamide, riboflavin contents were highest when the genotypes were cultivated in Le Moulon. In contrast, the proportion of pyridoxal and

pantothenic acid were greatest in Clermont-Ferrand. However, the content of pyridoxine was similar between locations. There was a striking increase in the mean contents of pantothenic acid (+ 62.5%) and a significant decrease in the mean contents of nicotinamide (-52 %) when accessions were grown in Clermont-Ferrand compared to the mean contents of the same accessions grown in Le Moulon. Even though statistically significant, the mean levels of thiamin and riboflavin in the genotypes cultivated in Le Moulon were only 12.5% and 6 % highest than those genotypes grown in Clermon- Ferrand. There was a significant difference between the two sites for average of TKW (thousand-kernel weight), with lowest TKW in Clermont-Ferrand (40.67 g/1000 kernels) and highest TKW in Le Moulon (47.10 g/1000 kernels). Empirical studies in France (Gate et al., 2010) have shown that each day of heat stress (maximum temperatures exceed 25°C) during grain filling, can be linked to average loss of circa 0.8 g of thousand kernel. It is therefore conceivable, that the period of drought stress (8 days without precipitation and maximum temperatures exceed 25°C) observed at Clermont-Ferrand during grain filling (Chapter 3, Table 3) may have contributed to the reduction of wheat yields in Clermont-Ferrand. Thus, if we used the model proposed by Gate et al. (2010) with our data, the predicted value of their model (8*0.8 = 6.4) is very similar to the value obtained when we substracted the average TKW in Le-Moulon and the average TKW in Clermont-Ferrand (47.10-40.67 = 6.43). Previous study of variation in bread wheat B vitamins among growing locations is scarce. In agreement with the present finding, Shewry et al. (2011b) recently observed significant effects of growing location on the B vitamin contents of 26 bread wheat lines grown on four sites in 2006-2007. Indeed, vitamins thiamin, niacin and B6 showed similar positive correlations with mean temperature between heading and harvest. For those vitamins, the mean contents were highest in the sample grown in Hungary where the precipitation was the lowest and the mean temperature between heading and harvest were the highest. Additionally, bread wheat lines grown in Hungary resulted in smaller kernel size than the same lines grown in the other locations (The HEALTHGRAIN Project). This observation is not totally consistent with our findings, because the highest average contents of thiamin, niacin, riboflavin were found in wheat genotypes grown in Le Moulon, where the average TKW was higher than in Clermont-Ferrand. Furthermore, the mean contents of those vitamins expressed by grain were slightly higher in Le Moulon. In contrast, the lowest average contents of pantothenic acid and pyridoxal by kernel (table 3) were observed in Le Moulon. Teply et al. (1942) also reported that environmental differences have considerable effects on the pantothenic acid contents of four varieties of dark hard winter wheat. From the results observed, it is therefore possible that environmental factor like drought stress (Chapter 3, Table 3), that occurred at Clermont- Ferrand in 2006, may have a detrimental impact on the content of B-vitamins. Little is known about the detailed distribution of bioavailable form of niacin and pyridoxine in wheat kernel. Taking into account that the average TKW was 14% lower in Clermont Ferrand than in Le Moulon , it is therefore conceivable that a fraction of those compounds are located in the endosperm and that any factors affecting the proportion of the endosperm will affect their concentrations in the whole grains. Another hypothesis could be that the wheat bran effectively provides an important part of those vitamins and thus smaller grains, which have higher proportions of outer layers and embryo relative to starchy

endosperm should have lead to increase the contents of those vitamins. However, the drought stress period occurring during grain filling, may have reduced the synthesis of those compounds, or may have consumed them in order to protect the grain development. Thus, a higher content of those vitamins would be found in larger kernel size which has not been stressed by environmental conditions. A similar pattern was not observed for pantothenic acid and pyridoxal. Indeed, the results showed that higher proportion of bran (whole grain flour made with smaller kernels are enriched in bran because smaller grains have a greater ratio of outer layers to starchy endosperm) have resulted in higher pantothenic acid and pyridoxal content by gram of flour. In addition, average contents of pantothenic acid and pyridoxal by kernel were higher in Clermont-Ferrand where the TKW was lower. Thus, it could mean that the bran may be more concentrate in pantothenic acid than the other parts of the grain. It is also possible that elevated levels of pantothenic acid and pyridoxal are synthesized in plants in extreme environmental conditions, such as water stress or high temperature, since pyridoxal compounds are well known to play a role in oxidative stress responses.

Table 3. Mean contents of B vitamins and TKW of the 195 wheat accessions cultivated in Le Moulon and Clermont-Ferrand[a].

	Le Moulon 2006-2007				Clermont-ferrand 2005-2006			
	Thiamin	Nicotinic acid	Nicotinamide	Pyridoxal	Thiamin	Nicotinic acid	Nicotinamide	Pyridoxal
av	5.096[a] (**0.24**)[c]	3.258[a] (**0.15**)	1.287[a] (**0.06**)	0.641[b](**0.03**)	4.446[b](**0.17**)	2.266[b](**0.1**)	0.484[b](**0.024**)	0.837[a](**0.04**)
SEM	0.022(**0.0003**)[d]	0.025(**0.0015**)	0.01(**0.0002**)	0.008(**0.0002**)	0.022(**0.0006**)	0.025(**0.0006**)	0.01(**0.0002**)	0.008(**0.0007**)
	Pyridoxine	Riboflavin	Pantothenic acid		Pyridoxine	Riboflavin	Pantothenic acid	
av	1.101(**0.05**)	0.879[a](**0.04**)	3.833[b](**0.18**)		1.071(**0.057**)	0.824[b](**0.03**)	8.033[a](**0.27**)	
SEM	0.012(**0.0002**)	0.015(**0.0002**)	0.004(**0.0003**)		0.012(**0.0001**)	0.015(**0.0002**)	0.004(**0.002**)	
	TKW				TKW			
[e]av	47.10[a]				40.67[b]			
SEM	0.14				0.14			
CV (%)	11.66				11.07			

TKW, thousand-kernel weight

av, average content (µg/g DM)

[a]Different letters following the average content for each B vitamin indicate a statistically differences between the two locations ($p<0.05$)

[c]Values in bold and in brackets refer to the average expressed in µg/grain

[d] Values in bold and in brackets refer to the standard error mean of the data expressed in µg/grain

[e]av, average content expressed in grams

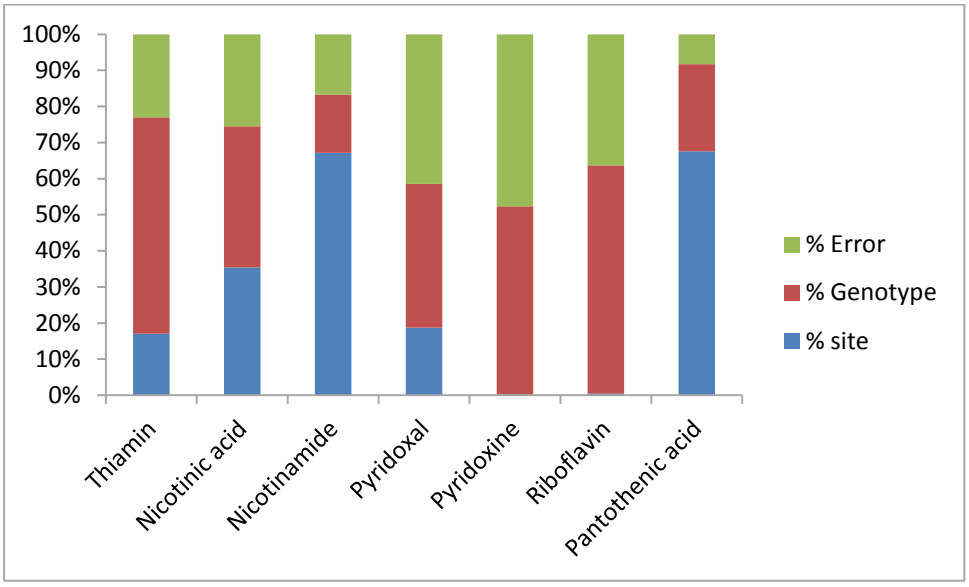
2.1.3. Effect of environment and variety on water-soluble vitamins in the bread wheat core collection

As shown in Figure 1, the genotype effect explained 60 and 63 % of the total variation for thiamin and riboflavin, respectively. A completely different pattern was observed for nicotinamide and pantothenic acid with environment accounting for about 70% and genotype for 16 % (nicotinamide) and 24 % (pantothenic acid). The variation in pyridoxine and riboflavin were only explained by the genotype and error (including GxE) effects. For nicotinic acid, 35 % of the variation was ascribed to environment, 39 % to genotype and 25 % to error. Shewry et al. (2011b) reported that vitamin B1,B3 and B6 was mainly affected by environment (50-70% of the variation), whereas genotype accounting for about 5% (vitamin B3), 10% (vitamin B6) and 27% (vitamin B1). The differences observed between our study and Shewry et al (2011b) might be mainly due to the analytical method used.

2.1.4. Heritability of contents of B vitamins

Heritability studies provide valid information about the traits that are transmitted from parents to successive generations. Such studies help plant breeders to use best genetic stock for improving the crop. The highest heritability was observed for thiamin (87%), nicotinic acid (78%), riboflavin (80%) and pantothenic acid(89%).Whereas heritability of 69 % were recorded for nicotinamide, pyridoxine and pyridoxal (table 1). The present finding suggest that the variation in B vitamin is highly heritable and that B vitamin contents could be substantially increased by breeding.

Figure 1. Percentage of the contributions of G, E and Error to the variability of the B-Vitamins for the 195 wheat accessions



The relative contributions of variances sources (G, E and error) were their sum square (SS) percentage of the total SS of the model.

2.2. Effect s of genotype and environment on vitamin E, Lutein and β-sitosterol contents in the bread wheat core collection

2.2.1. Variation in the α-tocotrienol, β-sitosterol and lutein contents of 195 bread wheat genotypes grown at two locations

In our study, bread wheat genotypes of the core collection were found to be very poor in α-tocotrienol with average value of 1.04 µg/g DM. When tocols profile was examined, it was found that β-γ-tocopherol and β-γ-tocotrienol were the major tocols present with average contents of 15.77 µg/g DM and 10.69 µg/g DM, respectively (Table 1). There were a 32-fold, 15.5-fold and 11.63-fold difference in bread wheat accessions in α-tocotrienol, α -tocopherol and β-γ-tocopherol contents respectively, indicating that a lot of variation was present. The extent of variation for β-γ-tocotrienol was smaller with a 7-fold difference between maximum and minimum values.The mean value for total tocol reported in our study was substantially lower (30.58 µg/g DM) than that reported by Lampi et al. (2008) on whole meal flours of 150 wheat lines (130 winter types and 20 spring types) grown in Hungary in 2005, which ranged the content of total tocol from 27.6 to 79.7 µg/g DM with an overall mean of 49.81µg/g DM. The effect can be explained by the fact that our extraction procedure did not include a saponification step which has been shown to provide significantly higher tocol recoveries (Panfili et al., 2003). Interestingly, when individual tocol contents were examined, the differences between our results and the ones described by Lampi et al. (2008) are mainly on α-tocopherol, α-tocotrienol, and β-γ-tocotrienol, which could mean that in bread wheat an important amount of these forms of tocols exist as tocols esters. In this, study, the determination of sterols was obtained without acid hydrolysis and subsequent saponification as suggested by Lafelice et al. (2009). Thus, only the free forms of sitosterol were examined in the different bread wheat genotypes. In hexaploid wheats the free sterol fraction is predominant and represents 51 % of total sterol (Lafelice et al., 2009). The average free sitosterol contents of 195 accessions varied from 4.58 to 228 µg/g DM, indicating a 50-fold difference in the phenotypic values measured on the two locations (Table 1). The present findings reveal the high variability for free sitosterol contents. Furthermore, the differences in free sitosterol contents were higher than the variation in total sitosterol contents reported by Numi et al. (2010). As presented in Table 1, there was a 88.5-fold difference in bread wheat accessions in the levels of lutein. The range was the greatest among the lipid-soluble compounds analysed in the bread wheat lines of the core collection. Nevertheless, the mean value was only 0.41 µg/g DM. As mentioned by Leenhardt et al. (2006), the level of this carotenoid is particularly low in bread wheat compared to the concentration found in diploid and tetraploid wheats. When the data of the two environments were considered, for each fat-soluble compounds contents, there were highly significant differences ($p<0.001$) between the genotypes (Table 4). These high genetic effects are therefore very important for future wheat breeding. The present findings are in accordance with the study of Ward et al. (2008). The large genetic effect observed for the

lipid-soluble compound contents also demonstrates analysed accessions for those metabolites and strengthen the usefulness of that core collection.

Table 4. Sum of squares of variance analysis for vitamin E, Lutein and β-sitosterol.

Source of variation	Degree of freedom	α-tocopherol	β γ-tocopherol	α-tocotrienol	β-γ-tocotrienol
Genotype	195	455.933*	7866.06*	65.7627*	5650.97*
Location	1	115.258*	10281.5*	105.283*	209.534*
Error	580	196.98	5071.99	23.9476	1950.96
		β-sitosterol	Lutein		
Genotype	195	496752*	76.8295*		
Location	1	129041*	1.12876*		
Error	580	116693	6.62173		

*P value <0.001.

2.2.2. Variation of fat compound contents between growing locations

Contrasted variation was observed in the fat compounds contents of 195 wheat genotypes grown at two locations. The mean values for all accessions per site are given in Table 5. The average level of fat-compounds was significantly different between locations as measured with the LSD test ($p < 0.05$). The average α-tocopherol, α-tocotrienol, β-γ-tocotrienol, β-sitosterol and lutein contents were highest when the accessions were cultivated in Le Moulon. In contrast, the level of β-γ-tocopherol was greater in Clermont-Ferrand. The differences among the average levels of β-γ-tocopherol, α-tocotrienol and β-sitosterol at the various locations were 37 %, 52 % and 34 %, respectively. Even though statistically significant, the average level of α-tocopherol and β-γ-tocotrienol in the genotypes cultivated in Le Moulon were only 6 % and 9 % higher than those genotypes grown in Clermont-Ferrand. High temperature and water stress that occurred at Clermont- Ferrand in 2006, resulted in smaller grains. Furthermore, there was a moderately strong negative correlation between β -γ-tocopherol contents and kernel size at both sites (Table 5). Hence, higher proportions of β -γ-tocopherol would be expected in genotypes which have smaller grains. Similarly, Lampi et al. (2008) observed a similar correlation ($r = -0.543$, $df = 128$, $p < 0.01$) between kernel size and total tocol contents in winter wheat genotypes grown in Hungary. These findings were quite expected, in view of the fact that many bioactive compounds are concentrated in the bran and the germ, and that the proportions of these fractions are greater in small kernels than in large kernels (Nyström et al., 2007). Interestingly, there was a relatively weak but statistically positive relationship between α-tocopherol, α-tocotrienol ,

lutein,β-sitosterol contents and TKW (Table 5) at Clermont-Ferrand , which were not in agreement with the previous finding of Nurmi et al. (2010) and Lampi et al. (2008). Nevertheless, it has to be noticed that in wheat, sterols and tocols can be found as free, esterified and conjugated with sugars (Lafelice et al., 2009). The study of Nurmi et al. (2010) and Lampi et al. (2008) have reported the significantly negative relationship between the total content of sterols and tocols (free and bounding forms) and kernel characteristics. Nevertheless, in our study, only the free forms were considered. Thus, our data suggested that kernels bigger in size tended to have greater levels of free sterol and free α-tocopherol, α-tocotrienol and lutein. Little is known about the detailed distribution of the different forms of tocols in wheat kernel. It is therefore possible, with the exception of β-γ-tocopherol, that an important part of the free form of lipid-soluble compounds in wheat grain are located in the endosperm of the kernels.

Table 5. Mean contents of vitamins E, Lutein and β-sitosterol and Pearson correlation coefficients (r) between vitamin contents (µg/g) and TKW in the 195 wheat accessions cultivated in Le Moulon and Clermont-Ferrand[a].

	Le Moulon 2006-2007			Clermont-Ferrand 2005- 2006		
	α-tocopherol	β-γ-tocopherol	α-tocotrienol	α-tocopherol	β-γ-tocopherol	α-tocotrienol
av	3.46[a] (**0.16**)[c]	12.11[b] (**0.57**)	1.41[a] (**0.067**)	2.69[b] (**0.11**)	19.46[a] (**0.79**)	0.67[b] (**0.028**)
SEM	0.03 (**0.002**)[d]	0.15 (**0.008**)	0.01 (**0.001**)	0.03 (**0.002**)	0.15 (**0.01**)	0.01 (**0.0006**)
CV (%)	25.63	28.47	26.95	35.31	23.91	43.82
TKW	r=-0.11	***r=-0.18***	r=0.058	***r=0.16***	***r=-0.39***	***r=0.39***
	P=0.038	***P < 0.001***	P=0.26	***P < 0.001***	***P < 0.001***	***P < 0.001***
	β-γ-tocotrienol	β-sitosterol	Lutein	β-γ-tocotrienol	β-sitosterol	Lutein
av	11.2[a] (**0.53**)	75.97[a] (**3.58**)	0.45[a] (**0.02**)	10.18[b] (**0.42**)	50.01[b] (**2.06**)	0.37[b] (**0.015**)
SEM	0.09 (**0.009**)	0.72 (**0.08**)	0.005 (**0.0008**)	0.09 (**0.007**)	0.72 (**0.05**)	0.005 (**0.0007**)
CV (%)	27.98	42.14	71.97	30.84	47.11	88.58
TKW	r=0.01	r=0.06	r=0.09	r=0.0812	***r=0.26***	***r=0.12***
	P=0.05	P=0.21	P=0.06	P=0.026	***P < 0.001***	***P < 0.001***

TKW, thousand-kernel weight

av, average content (µg/g DM)

[a] Different letters following the average content for each fat-vitamin indicate a statistically differences between the two locations ($p<0.05$)

Value in bold italics are statistically significant (P-value < 0.01)

[c]Values in bold and in brackets refer to the average expressed in µg/grain

[d] Values in bold and in brackets refer to the standard error mean of the data expressed in µg/grain

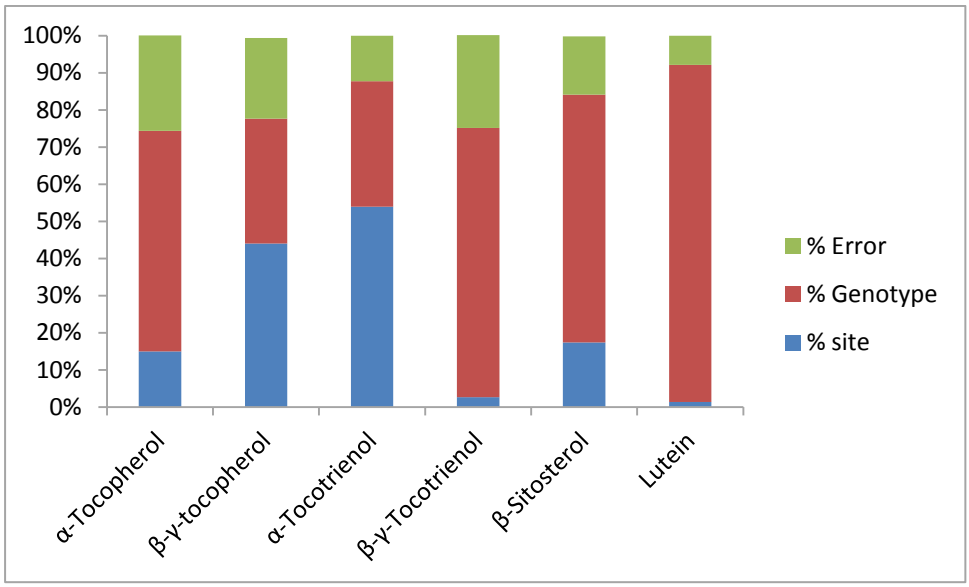
2.2.3. Effect of environment and variety on lipid-soluble compounds in the bread wheat core collection

As shown in Figure 2, the genetic effect on the contents of α-tocopherol, β-γ-tocotrienol, β-sitosterol and lutein was the greatest. A completely different pattern was observed for β-γ-tocopherol and α-tocotrienol with environment accounting for about 44% and 54% respectively. This result was in agreement with Shewry et al. (2010), who reported that the ratio of genotypic variance to total variance for total tocols and total sterols was high (0.77 and 0.57 for tocols and sterols, respectively). For β-γ-tocopherol and α-tocotrienol, the high percentage of variation attributed to the environment indicates that the numerous genes involved in the quantitative expression of those metabolites are strongly influenced by environmental factors.

2.2.4. Heritability of contents of lipid-soluble compounds

The heritabilities were high with 97% for lutein content, 92% for β-sitosterol and around 80% for tocols (table 1). Such a high heritability indicates that developing new wheat cultivars with high contents of lipid-soluble compounds could be achieved by classical plant breeding.

Figure 2. Percentage of the contributions of G, E and Error to the variability of the lipid-soluble compounds for the 195 wheat accessions



The relative contributions of variances sources (G, E and Error) were their sum square (SS) percentage of the total SS of the model.

2.3. B and E vitamin, Lutein, β-sitosterol contents variation among release date and geographical origin of wheat genotypes

2.3.1. The contents B and E vitamin, Lutein, β-sitosterol have not decreased with modern plant breeding

Since the middle of the 20th century, there have been clearly major changes in agronomic practices, such as increased use of fertilizers, crop protection chemicals as well as the free and widespread global exchange of wheat germplasm. Furthermore, agronomic practices also vary between the countries of origin, with varieties being selected and grown under higher input conditions in Western Europe than in most other regions (Shewry et al., 2011). Therefore, it was useful to evaluate the variability in the content of vitamins of modern varieties (registered after 1960) with those of other accessions registered before 1960, ie. landraces and old varieties. In addition, the diversity in vitamin contents of Western European genotypes was compared with genotypes from other regions of the world.

There was a weak tendency for the average level (table 6) of individual B-vitamins in modern accessions to be significantly ($p < 0.05$) lower than in landraces and old varieties. However, these differences were more marked in the location of Le Moulon (Figure. 3). Box plot distributions of B-vitamin contents given in figure 3 provided evidence that variations among the two groups of genotypes (modern vs other accessions) were at comparable levels. TKW and kernel number per square meter are the two main indicators of yield. It has been suggested that improvements in breeding new cultivars have mainly resulted from a greater ability to fill an increasing number of kernels (Brancourt-Hulmel et al., 2003). Nevertheless, in our study the modern varieties showed higher TKWs and a much larger range of variation than other accessions when grown (in Le Moulon) under the same agronomic and environmental conditions (Figure 3).

The variability of lipide-soluble compound contents was not totally consistent with the tendency observed for the water soluble vitamins. Indeed, there was a large variation for each vitamin within each varieties group. Modern genotypes were found to be significantly ($p<0.05$) richer in tocotrienols (Table 7) and also presented broader variation than landraces and old accessions (Figure. 4). Thus, the modern genotypes contribute much of the diversity in tocotrienols of the core collection. On the contrary, there was a weak but significant ($p<0.05$) tendency for other accessions class to accumulate more β-γ-tocopherols (Table 7). There was not, however, a statistically significant difference ($p <0.05$) in β-sitosterol and lutein contents among the two wheat groups in both locations (Table 7). Meanwhile, box plot distributions of lutein for both groups in Le Moulon were significantly smaller than in Clermont-Ferrand (Figure. 4). Hence, these data indicated that content of lutein (mainly present in endosperm) varies considerably from site to site, and that factors such as temperature may significantly influence the phenotypic diversity in lutein content as it was revealed here for the core collection. The average lutein content (in µg/g DM) was higher at Le Moulon. Thus, as suggested by Shewry et al. (2010), “it will be interesting to source material grown in environment that results in higher levels of specific bioactive compounds,

for example to exploit the higher levels of folates, tocols, sterols, and phenolic acids present in grain grown at higher temperatures". Cultivation of the core collection in dry area may facilitate the identification of lines with high content of lutein or for incorporation into breeding programs as sources of high levels of lutein.

Interestingly, if the accessions were ranked according to their contents of B and E vitamin, Lutein and β-sitosterol as described by Ward et al. (2008), no significant correlations were found between release date and group of vitamin contents (Figure. 5). Our results are in accordance with those previously described within the HEALTHGRAIN diversity screen (Shewry et al., 2012), which indicated that using a limited number of cultivars that the contents of bioactive components have not decreased with modern plant breeding.

Table 6. Mean contents of B vitamins of modern varieties and other accessions of the core collection cultivated in Le Moulon and Clermont-Ferrand[a,b].

Vitamins (μ/g)	Cultivation year/ Location and Modern varieties or other accessions			
	2006/2007-LeMoulon		2005/2006-Clermont-Ferrand	
	Older accessions	Modern cultivars	Older accessions	Modern cultivars
Thiamin	5.34 ± 0.06[a]	4.91 ± 0.05[b]	4.84 ± 0.06[a]	4.20 ± 0.04[b]
Nicotinic acid	3.43 ± 0.06[a]	3.17 ± 0.06[b]	2.30 ± 0.04	2.25 ± 0.03
Nicotinamide	1.36 ± 0.03[a]	1.24 ± 0.03[b]	0.5 ± 0.008[a]	0.47 ± 0.008[b]
Pyridoxal	0.65 ± 0.02	0.64 ± 0.02	0.86 ± 0.015[a]	0.81 ± 0.013[b]
Pyridoxine	1.11 ± 0.03	1.08 ± 0.02	1.10 ± 0.02	1.04 ± 0.02
Riboflavin	0.96 ± 0.04[a]	0.86 ± 0.04[b]	0.8 ± 0.02	0.85 ± 0.03
Pantothenic acid	4.41 ± 0.1[a]	3.63 ± 0.06[b]	8.32 ± 0.18	7.92 ± 0.11

[a]Results are expressed as mean ± SEM on a dry weight basis

[b]Different letters following the average content for each B vitamin indicate a statistically differences between older accessions and modern cultivars ($p<0.05$)

Table 7. Mean contents of lipid-soluble compounds of modern varieties and other accessions of the core collection cultivated in Le Moulon and Clermont-Ferrand[a,b].

Compounds (μ/g)	Cultivation year/ Location and Modern varieties or other accessions			
	2006/2007-LeMoulon		2005/2006-Clermont-Ferrand	
	Older accessions	Modern cultivars	Older accessions	Modern cultivars
α-tocopherol	3.5 ± 0.07	3.39 ± 0.06	2.65 ± 0.08	2.67 ± 0.068
β -γ-tocopherol	12.59 ± 0.31[a]	11.53 ± 0.024[b]	19.96 ± 0.38[a]	18.62 ± 0.31[b]
α-tocotrienol	1.33 ± 0.025[b]	1.45 ± 0.03[a]	0.6 ± 0.02[b]	0.71 ± 0.023[a]
β-γ-tocotrienol	10.65 ± 0.20	11.26 ± 0.25	9.63 ± 0.25[b]	10.40 ± 0.23[a]
β-sitosterol	76.85 ± 2.72	78.80 ± 2.42	49.95 ± 1.92	50.31 ± 1.73
Lutein	0.43 ± 0.02	0.48 ± 0.028	0.35 ± 0.02	0.42 ± 0.028

[a]Results are expressed as mean ± SEM on a dry weight basis.

[b]Different letters following the average content for each compound indicate a statistically differences between older accessions and modern cultivars ($p<0.05$).

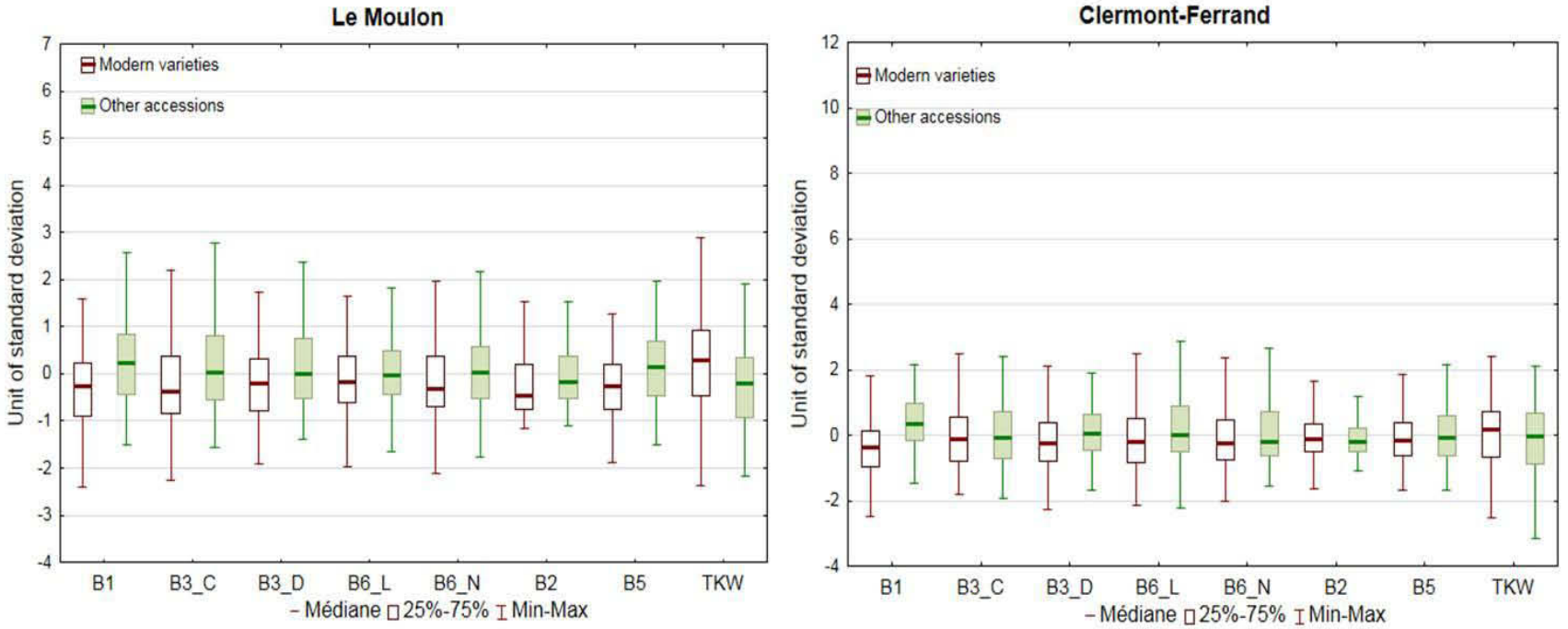


Figure 3. Box plot graph of modern varieties and other accessions of the core collection for thiamin (B1), nicotinic acid (B3_C), nicotinamide (B3_D), pyridoxal (B6_L), pyridoxine (B6_N), riboflavin (B2), pantothenic acid (B5) contents and thousand kernel weight (TKW) parameter.

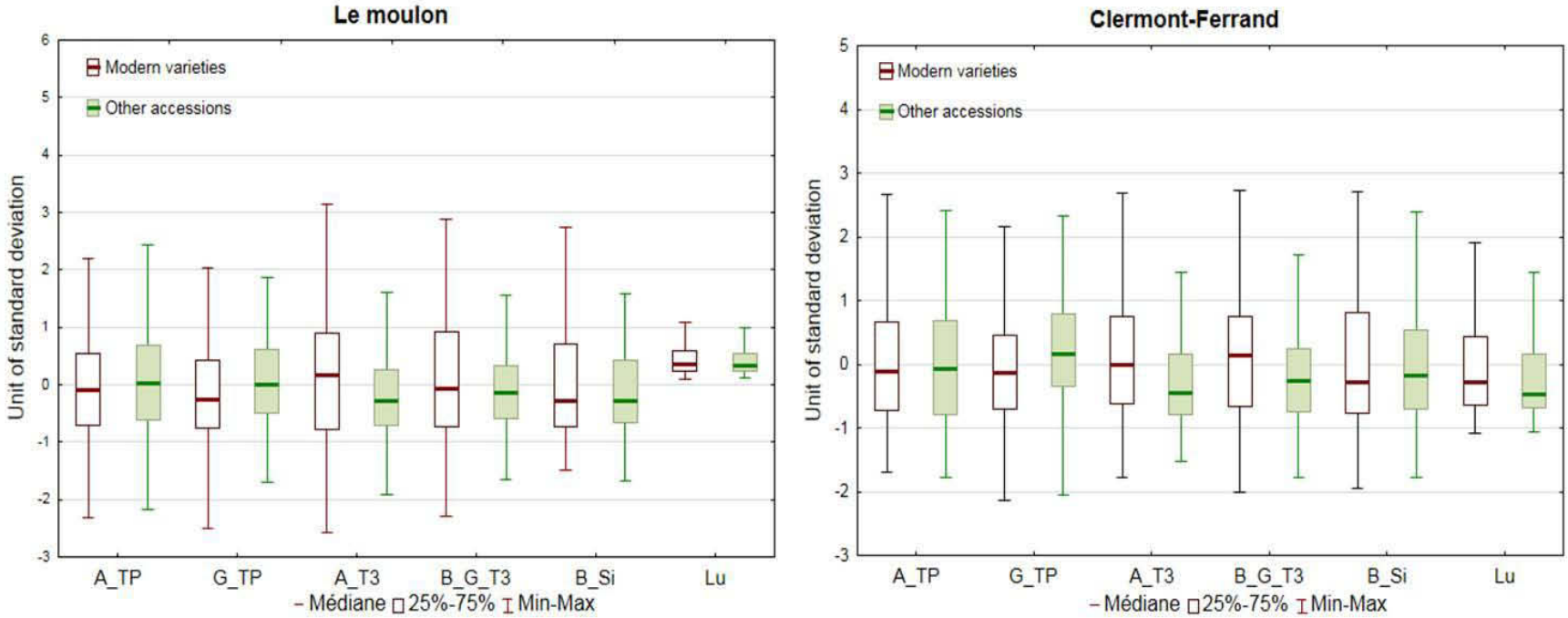


Figure 4. Box plot graph of modern varieties and other accessions of the core collection for α-tocopherol (A_TP), β-γ-tocopherol (G_TP), α-tocotrienol (A_T3), β-γ-tocotrienol (B_G_T3), β-sitosterol (B_Si), Lutein (Lu) contents.

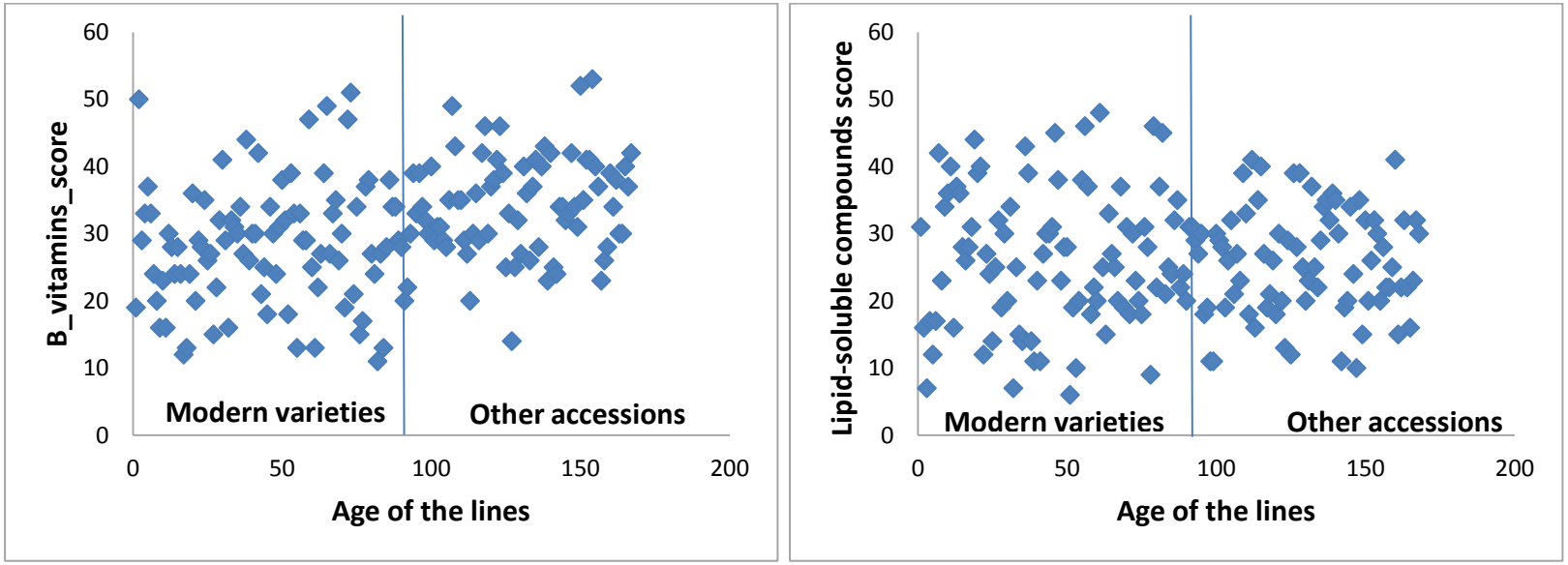


Figure 5. Relationship between release date and the contents of bioactive compounds (expressed as B-vitamins and lipid-soluble compounds score) for 173 wheat accessions. To calculate scores for bioactive compounds, the accessions were grouped into 8 classes for each compound, with group 8 comprising the 25 accessions with the highest contents and group 1 comprising the 25 accessions with the lowest contents. Addition of the scores for the individual compounds means that accessions with the highest levels of all B-vitamins or lipid-soluble compounds would have a maximum score of 56 and 48 respectively. From Shewry et al. (2012).

2.3.2. The contents of B and E vitamin, Lutein and β-sitosterol presented contrasted variation in relation to Western European and non-Western European genotypes in the core collection.

Box plot distributions of B-vitamins, whose range of Western European accessions was comparable to the accession's range of other regions, are given in figure 6. The average total thiamin and riboflavin contents of genotypes from non-western regions (other regions) were greater in both location for thiamin and only in Le Moulon for riboflavin. However, the range of nicotinic acid was greater in Western European genotypes cultivated in Le Moulon, indicating that these genotypes might be valuable to plant breeders for elevating nicotinic acid contents in wheat when growing condition were optimal. Nevertheless, a strong increase in the inter-quartile range of nicotinic acid was observed when genotypes from other regions were cultivated at high temperature (Figure. 6). This may suggests that the geographical origin and the environmental condition could influence the nicotinic acid levels, and the trend might be that accessions from other regions are better adapted, maintaining high levels of this vitamin at elevated temperature.

As presented in figure. 7, the lipid-soluble compounds were more abundant in Western European genotypes and among the two locations. In addition, Western European genotypes have a broader variation in β-γ-tocotrienol, β-sitosterol and lutein contents than non-Western European genotypes. A similar trend was also found in a study on winter and spring wheat accessions, where the best tocol sources were genotypes from western parts of Europe (Lampi et al., 2008).

The results show that independently of the geographical origin, there was a large variation in lipid-soluble compounds. Moreover, modern varieties from the western parts of Europe which seemed to be well adapted to environmental factor such as higher temperature might be potential reservoir of genotypes for improving the proportion of lipid-soluble compounds in wheat.

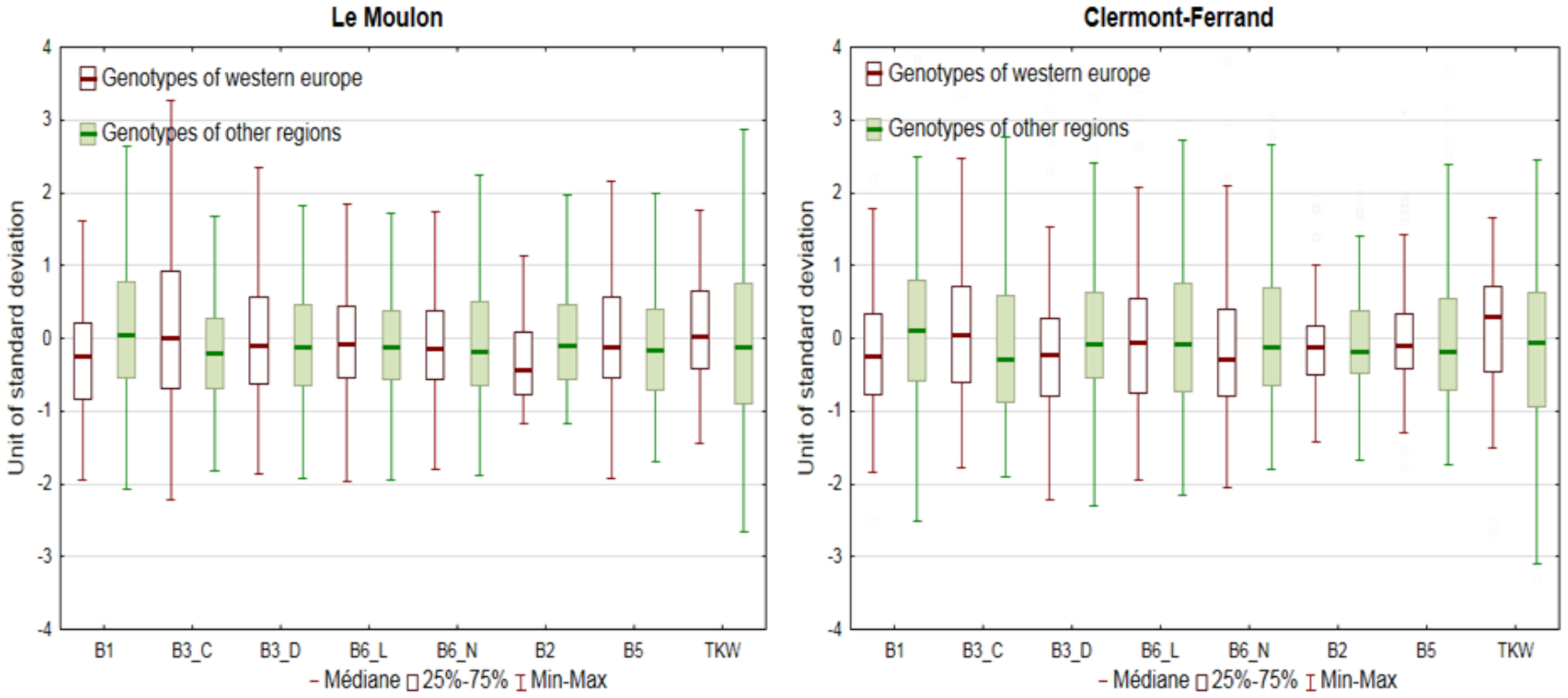


Figure 6. Box plot graph of western genotypes and of other regions of the core collection for thiamin (B1), nicotinic acid (B3_C), nicotinamide (B3_D), pyridoxal (B6_L), pyridoxine (B6_N), riboflavin (B2), pantothenic acid (B5) contents and thousand kernel weight (TKW) parameter.

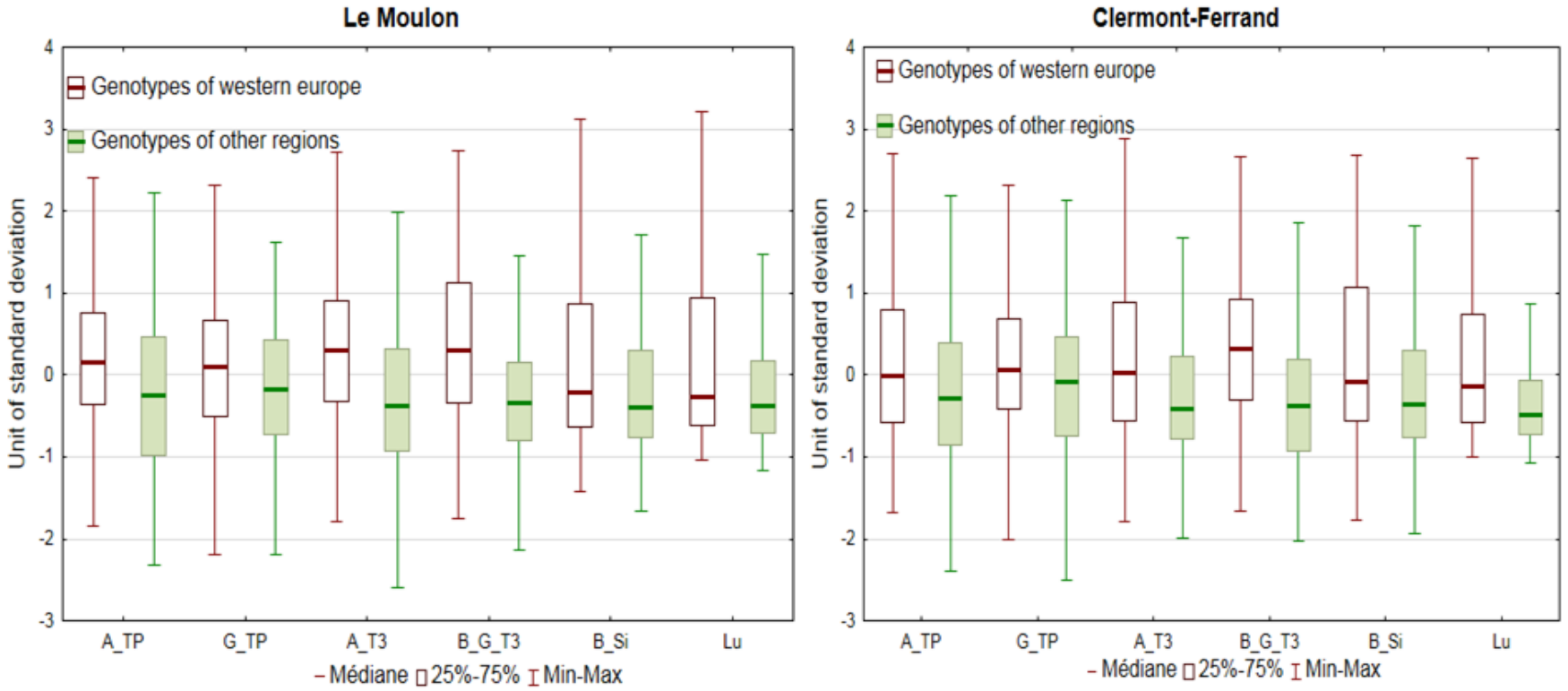


Figure 7. Box plot graph of western genotypes and of other regions of the core collection for α-tocopherol (A_TP), β-γ-tocopherol (G_TP), α-tocotrienol (A_T3), β-γ-tocotrienol (B_G_T3), β-sitosterol (B_Si), Lutein (Lu) contents.

2.4. Correlations between groups of bioactive components

2.4.1. Correlations between contents of B vitamins

Correlations between the mean contents of the B vitamins are given in table 8 and were calculated within each site to remove the influence of the environment. The contents of thiamin and pyridoxine showed positive correlations in the samples from Le Moulon but higher positive correlation when the samples from Clermont-Ferrand were analyzed. Furthermore, the contents of the nicotinic acid and nicotinamide were both weakly correlated with thiamin. Positive correlations with pyridoxine and pyridoxal were observed for nicotinamide and nicotinic acid at Clermont-Ferrand. Similarly, weak correlations between riboflavin and pyridoxine and pyridoxal were observed in samples grown in Le Moulon and Clermont-Ferrand. Shewry et al. (2011b) have also reported correlations between the contents of vitamin B1 and B3, B1 and B6 and B2 and B6. There are no direct known relationships between the biosynthetic pathways for these four B vitamins. Thus, those correlations between groups of B vitamins do not imply a direct relationship and they are likely to result from indirect effects which have to be determined. It is known that thiamin is formed by the condensation of hydroxyethylthiazole phosphate (HET-P) and hydroxymethylpyrimidine pyrophosphate (HMP-PP). The HET-P biosynthesis pathway in plants is assumed to be similar to that of yeast, in which HET-P synthase catalyzes the formation of the thiazole moiety from NAD+, glycine and sulfur from a donor (Belanger et al., 1995). Recently, it was shown that during the biosynthesis of HET-P, NAD+ was consumed as a substrate, releasing nicotinamide as a by-product (Chatterjee et al., 2007). Nicotinamide and nicotinic acid are both precursors for NAD+ biosynthesis via the salvage pathway. Therefore, it could be that the indirect effect explaining the correlation between vitamin B1 and B3 may be related to the consumption of NAD+ and the release of nicotinamide during the biosynthesis of thiamin. In addition, it was reported that in yeast cells, HMP-P is derived from histidine and vitamin B6, catalyzed by thiamine (THI5) protein (Coquille et al., 2012). Thus, if homolog of the protein THI5 was present in wheat, it could explain the link between the synthesis pathway for thiamin and vitamin B6.

2.4.2. Correlations between contents of vitamins E, Lutein and β-sitosterol

There was a strong correlation between lutein and β-sitosterol contents which was consistent at each location as shown in Table 9. In addition, α-tocopherol, α –tocotrienol and β-γ-tocotrienol were positively correlated with Lutein which is in agreement with Lv et al. (2013). The contents of tocopherols and tocotrienols were correlated positively with the contents of β-Sitosterol in samples grown in Le Moulon and Clermont-Ferrand. The respective correlations were statistically significant. These values indicate that with higher sterol contents, the tocol contents also tended to be higher. This relationship was also shown earlier in all bread wheat genotypes of the HEALTHGRAIN wheat diversity screen (Lampi et al., 2008; Shewry et al., 2010). However, this is the first report showing a strong relationship between lutein and sterol contents in bread wheat genotypes. Thus, the levels

of the three classes of lipid-soluble compounds seem to be strongly related, which is in accordance with the observation that many bioactive compounds are similarly located in wheat endosperm.

2.4.3. Correlation of wheat B-vitamins and lipid-soluble compounds with its protein content

A strong and consistent positive correlation was observed in both locations between thiamin and protein contents (Table 8). Those findings are in accordance with the investigations of Rohi et al. (2013) who reported that "with respect to whole wheat flour, a positive correlation has been detected for protein content with thiamin (r = 0.54)". It is suggested in the literature (Mitsunaga et al., 1986) that thiamin-binding proteins (TBP) which are found in many kinds of plant seeds retain thiamin in dormant seeds and supply thiamin for germ growth during seed germination. Watanabe et al. (2004) investigated the changes of globulin TBP activity and thiamin levels during wheat seed maturation. The results showed that globulin TBP activity increased in wheat seeds during maturation after flowering and might accumulate in the aleurone layer of the wheat seeds. As mentioned in chapter 4, thiamin was mostly accumulated in the enriched fraction. Even if more study have to be done about the proportions of the different grain tissues (aleurone, pericarp or germ) that compose the technological enriched fraction, the hypothesis about hight proportion of aleurone layers present in this fraction could be advance and thus, the strong positive correlation between thiamin and protein contents found in our study could be possibly explained by the presence of the globulin thiamin-binding protein in the aleurone layers of the different wheat seeds of the core collection. As mentioned above in section 2.1.3, the genotype effect explained 60% of the total variation of thiamin contents. Therefore, the synthesis and accumulation of thiamin or TBP in the peripheral cells containing the aleurone layer of wheat seeds could be under genetic control. Among all wheat genotypes and for both growing locations, the moderately strong correlation between α-tocopherol, β-γ-tocotrienol, sitosterol, lutein contents and protein contents were negative (Table 9). A strong negative correlation was also found between α-tocotrienol and protein contents ($r = -0.5311$, $p < 0.0001$ and $r = -0.4186$, $p < 0.0001$ at Clermont-Ferrand and Le Moulon respectively). Nevertheless, there was a relatively weak but statistically positive correlation between β-γ-tocopherol and protein contents at Clermont-Ferrand ($r=0.2889$, $p < 0.0001$). These suggest that apart from β-γ-tocopherol, the increase of lipid-soluble compounds level might negatively influence the grain protein content. Protein content is significantly negatively associated to the TKW, primarily resulting from starch accumulation. Hence, the higher the starch accumulation (lower protein content) the higher the contents of α-tocopherol, α-tocotrienol, β-γ-tocotrienol, sitosterol and lutein. This also suggests that β-γ-tocopherol might be mainly present in the aleurone layer and might not be influenced by the starch accumulation.

Table 8. Correlations among the contents of B-vitamins and B-vitamins and protein contents[a] in the bread wheat genotypes of the core collection grown in Le Moulon in 2005-2006 and in Clermont-Ferrand in 2006-2007.

	r (p)	
	Clermont-Ferrand	Le Moulon
	Thiamin	
Seed Vitamins (µg/g of DM)		
Pyridoxine	**0.3527 (<0.0001)**	**0.1322(0.009)**
Nicotinic acid	**0.1094 (0.0316)**	**0.1617 (0.0014)**
Nicotinamide	**0.1275 (0.0122)**	0.0711 (0.1610)
Technological trait		
Proteins content	**0.5711 (<0.0001)**	**0.4062 (<0.0001)**
	Nicotinic acid	
Seed Vitamins (µg/g of DM)		
Nicotinamide	**0.3208 (<0.0001)**	**0.4466 (<0.0001)**
Pyridoxal	**0.4522 (<0.0001)**	**0.1458 (0.0039)**
Pyridoxine	**0.2943 (<0.0001)**	**0.1265 (0.0124)**
Technological trait		
Proteins content	**0.1696 (0.0008)**	**0.1262 (0.0126)**
	Nicotinamide	
Seed Vitamins (µg/g of DM)		
Pyridoxal	**0.3036 (<0.0001)**	0.0764 (0.1318)
Pyridoxine	**0.2754 (<0.0001)**	**0.1265 (0.0124)**
Technological trait		
Proteins content	**0.2006 (0.0001)**	**0.0994 (0.0498)**
	Pyridoxal	
Seed Vitamins (µg/g of DM)		
Pyridoxine	**0.6175 (<0.0001)**	**0.7309 (<0.0001)**
Riboflavin	**0.1284 (0.0116)**	**0.1673 (0.0009)**
Technological trait		
Proteins content	**0.2987 (<0.0001)**	0.0403 (0.4274)
	Pyridoxine	
Seed Vitamins (µg/g of DM)		
Pantothenic acid	**0.2491 (<0.0001)**	**0.1110 (0.0284)**
Riboflavin	**0.1763 (0.0005)**	0.0467 (0.3576)
Technological trait		
Proteins content	**0.3182 (<0.0001)**	0.0734 (0.1480)

[a]Values depicted in bold represent a significant ($p<0.05$) correlation.

Table 9. Correlations among the contents of lipid-soluble compounds and lipid-soluble compounds and protein contents[a] in the bread wheat accessions of the core collection grown in Le Moulon in 2005-2006 and in Clermont-Ferrand in 2006-2007.

	r (p)	
	Clermont-Ferrand	Le Moulon
	α-Tocopherol	
Technological trait		
Seed Protein (%)	**-0.22 (<0.0001)**	-0.063 (0.2175)
Seed Compounds (µg/g of DM)		
β -γ-Tocopherol	-0.0013 (0.905)	**0.4006(<0.0001)**
α-Tocotrienol	**0.5967 (<0.0001)**	**0.4792(<0.0001)**
β-Sitosterol	**0.4170(<0.0001)**	**0.3218(<0.0001)**
Lutein	**0.2158 (<0.0001)**	**0.1777(0.0004)**
	β-γ-Tocopherol	
Technological trait		
Seed Protein (%)	**0.2889 (<0.0001)**	-0.0411(0.4190)
	α-Tocotrienol	
Technological trait		
Seed Protein (%)	**-0.5311 (<0.0001)**	**-0.4186 (<0.0001)**
Seed Compounds (µg/g of DM)		
β-γ-Tocotrienol	**0.5722 (<0.0001)**	**0.6949 (<0.0001)**
β-Sitosterol	**0.5084 (<0.0001)**	**0.3111 (<0.0001)**
Lutein	**0.2988 (<0.0001)**	**0.2968(<0.0001**)
	β-γ-tocotrienol	
Technological trait		
Seed Protein (%)	**-0.3763 (<0.0001)**	**-0.3485 (<0.0001)**
Seed Compoundss (µg/g of DM)		
β-Sitosterol	**0.3371 (<0.0001)**	**0.2233 (<0.0001)**
Lutein	**0.3810 (<0.0001)**	**0.3605 (<0.0001)**
	β-Sitosterol	
Technological trait		
Seed Protein (%)	**-0.2914 (<0.0001)**	**-0.1531 (0.0025)**
Seed Compounds (µg/g of DM)		
Lutein	**0.622 (<0.0001)**	**0.6633 (<0.0001)**
	Lutein	
Technological trait		
Seed Protein (%)	**-0.2063 (<0.0001)**	**-0.1818 (0.0003)**

[a]Values depicted in bold represent a significant (p<0.05) correlation.

2.5. Selection of wheat genotypes with high contents of bioactive components and high yields

The core collection has provided an important database on the content and composition of vitamins, as well as other indicators of quality traits in bread wheat. Combination of these data allows the selection of accessions in which high levels in vitamin B and E as well as Lutein and β-sitosterol are combined with good agronomic performance yield and with high quality for processing (TKW and protein content). To do this, the accessions were ranked according to their contents of B and lipide-soluble compounds as described by Ward et al. (2008). Accessions with the highest scores (i.e. highest amounts) for these components are listed in Table 10A. The data in Tables 10A and 10B are based on material grown on two sites, thus it highlighted the accessions which performed well in two different environments. The availability of data for TKW and protein content also allow us to restrict the selection to accessions that have high TKW and protein content in addition to high bioactive components, as shown in Table 10B. It is evident according to the results that within the core collection, it is possible to select lines which combine high levels of bioactive compounds with high yield and good quality traits.

Table 10. Top performing cultivars/accessions across two locations.

(A) Accessions with high contents of B-vitamins, lipid-soluble compounds and B + lipid-soluble compounds

Accessions with high content of B-vitamins (total B-vitamins ranking 39-53)		Accessions with high content of lipid-soluble compounds (total fat-vitamins ranking 39-48)		Accessions with high content of B and lipid-soluble compounds
BLE DE REDON BLANC 1/2	EBRO	BELLOVAC	ARTOIS-DESPREZ	BLANC PRECOCE
BLE DE REDON GLUMES VELUES 1	BLE D'OR	CH62022	GELPA	
AENT	BAIONETTE I	Danubia	CADENZA	
TOM THUMB	CROISEMENT 268	HIVERNAL	KID	
SPIN, 121-VAR,12/536	IAS 1	AMIFORT	DI6402-34-2-4	
BIITTA	BLE DANOIS	ATUT II	CORSODOR	
KIRAC 66	DI276	DI182-9	BLANC PRECOCE	
TURDA-81-77	STUBES DICKKOPF	MASTER	MIRLEBEN	
BLONDYNKA	BLE DE REDON BLANC BARBU 1 1	GRENIER	P, DE BROLLON	
ROUGE DE MARCHISSY	DETENICKA CERVENA	ROUGE-D'ALTKIRCH	KOLBEN 3	
DI15	NON_PLUS_EXTRA			
WS-13 CARDENO 34/45	BARBU DU FINISTERE			
BLANC PRECOCE	DI182-9			
ESPOIR	CHORTANDINKA			
FRUH-WEIZEN	CENAD 512			
CH01193	DIANA			
E108	ORFIELD			
INSTITUT 1802	BLE DE HAIE			
CANDEAL DE AREVALO	CP4			
DI185				

Table 10. Top performing cultivars/Accessions across two locations.

(B) Accessions with high contents of B-vitamins, lipid-soluble compounds and B + lipid-soluble compounds combined with Protein contents above 14% and Thousand kernel weights above 40g (DM)

Accessions with high content of B-vitamins (total B-vitamins ranking 39-53)		Accessions with high content of fat-vitamins (total fat-vitamins ranking 39-48)	Accessions with high content of B and fat vitamins
BLE DE REDON BLANC 1/2 LACHE 1 1	BLE DANOIS	CH62022	BLANC PRECOCE
BLE DE REDON GLUMES VELUES 1	BLE DE REDON BLANC BARBU 1 1	ROUGE-D'ALTKIRCH	
TOM THUMB	CENAD 512	GELPA	
BLONDYNKA	DIANA	BLANC PRECOCE	
ROUGE DE MARCHISSY	CP4		
DI15			
BLANC PRECOCE			
ESPOIR			
FRUH-WEIZEN			
E108			
INSTITUT 1802			
CANDEAL DE AREVALO			
DI185			
EBRO			
CROISEMENT 268			

3. Conclusion

This study provides new data on variation in bioavailable niacin and pyridoxine, thiamin, riboflavin, pantothenic acid as well as lutein and the free fraction of sterols and tocols contents of bread wheat. The very large number of genotypes grown on two different locations provided a unique opportunity to describe the core collection in term of nutritional traits and to estimate the genetic variation and relationship between bioactive compounds and kernel characteristics. The analyses reported here will have significant impact for plant breeders and food industries in the objectives of developing new cereal products with enhanced nutritional benefits. The results showed that genotype has a significant impact on the variations of nicotinamide, riboflavin and pantothenic acid contents for the B-vitamins and significant impact on the variations of α-tocotrienol, β-sitosterol and lutein contents for the lipid-soluble compounds, which is indicative of the wide diversity of the core collection. The range of variation in lutein contents was the greatest among the lipid-soluble compounds. Growing environment may significantly affect the vitamin compositions. For instance, higher temperature during the grain-filling phase, seemed to improve the average contents of pantothenic acid and β-γ-tocopherol. In addition, higher content of β-γ-tocopherol and lower content of α-tocotrienol were expected in genotypes which have smaller grains. Because all accessions of the core collection were grown in two environments, we were able to partition the variations in contents of B and lipid-soluble compounds between genotype, environment and a residual which included genotype x environment (G x E). Furthermore, it was suggested that within the core collection, the variation in B and lipid-soluble compounds was highly heritable. Thus, it might be possible to find genotype that could be potential candidates for the breeding in Europe of stable and high concentrations of bioactive compounds in bread wheat cultivars.

The evaluation of the relationship between the contents of vitamins and the year of release or geographical origins of wheat accessions in the core collection, has led to the conclusion that the contents of vitamins have not decreased with modern plant breeding. Moreover, modern varieties from the western parts of Europe might be potential reservoir of genotypes for improving the proportion of lipid-soluble compounds in wheat.

The size, shape, weight and color of kernels determine the commercial value of wheat grains. These features are associated with its technological and baking value of the flour. In general, big grains containing large amounts of nutrients, with uniform color and dimensions, are more desirable for food processing. Our data suggested that drought stress, which resulted in smaller kernel, have a detrimental impact on the levels of thiamin, riboflavin and bioavailable form of niacin and pyridoxine as well as free sterol and free α-tocopherol, α-tocotrienol and lutein.

The analysis of the extent of variation in the contents of vitamins and other grain traits in the core collection has permitted, however to select accessions combining high levels of vitamins with high thousand kernel weight and protein content.

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Association study for the B and E vitamin and Lutein, β-sitosterol contents of flour in a worldwide bread wheat core collection

1. Introduction

World wheat production in 2015 is anticipated to reach 724.3 million tons, a slightly increase compared to 2014 (Source: FAO, 2015). Bread wheat (*Triticum aestivum*) accounts for around 95% of the wheat grown in the world, with most of the remainder being Durum wheat (*T. durum*) (Peng et al., 2011) and participates for 20% of the calories consumed by humans. The grain of wheat is milled into flour and is subsequently processed into a variety of foods that have important roles in human nutrition, such as bread, steamed bread or noodles. However, most of the products are produced from refined wheat flour ,which is inferior to whole grain flour in its contribution to nutritional and. Epidemiological studies have clearly shown that whole-grain cereals can protect against obesity, diabetes, and cancers (Fardet, 2010). Even if the recommended consumption of whole grain products differed from one country to another, most of them recommend an increase in cereal consumption with emphasis on whole-grain products (Lang et al., 2003). The bran and germ fractions of the wheat grain contain most of the B-and fat-soluble vitamins. Those micronutrients are needed in small quantities for the normal functioning of the human body and have to be supplied through the diet because they are not synthesized by the human organism. Vitamin content varies greatly among different varieties of wheat (Chapter 6 of this document). We have also reported in the previous chapter correlations between the contents of vitamin B1 and B3, B1 and B6 and B2 and B6, as well as associations between the three classes of liposoluble compounds. Abundance of these flour nutritional-related traits is influenced by both genetic and environmental factors and some of them exhibit high genotype x environment interaction. Thus vitamin contents in bread wheat are quantitative traits controlled by a complex genetic system and for some vitamins environmental factors would play an important influence. Quantitative trait locus (QTL) analysis has provided an effective approach for the identification of polymorphic markers associated with phenotypic variation. Such tool aims to facilitate the understanding and the dissection of genome's regions that affect complex traits (Doerge, 2002). A large number of QTLs have been focused on flour color (Blanco et al., 2011; Roncallo et al., 2012) and yellow pigment contents (Patil et al., 2008; Blanco et al., 2011; Roncallo et al., 2012). Some QTL studies have focused on the genetics of accumulation of micronutrients like phytate, minerals, lutein or beta-carotene in the grains of wheat and rice (Stangoulis et al., 2007; Shi et al., 2008; Peleg et al., 2009; Roshanzamir et al., 2013; Zhao et al., 2013). Until a few years ago, the traditional method to identify QTL in plants involved the development of a segregating population from two genotypes varying in phenotypic values for a trait of interest. Using this approach, QTL identification was limited to loci that differed between the parents, and unless large populations were used, QTL with small effects could not be detected (Buckler and Thornsberry, 2002). Association mapping (AM) is a

complementary strategy to QTL mapping. The basic objective of AM, which is based on the concept of linkage disequilibrium (LD), is to detect correlations between genotypes and phenotypes in a sample of unrelated individuals. Therefore, compared to traditional QTL mapping, AM can be used to identify QTL in a much broader populations and hence mapping at higher resolution (Yu and Buckler, 2006). To the best of our knowledge, there is a lack of published literature on genome-wide AM studies of bioactive component traits in bread wheat. Reimer et al. (2008) identified by AM novel genomic regions associated with yellow pigment in an elite collection of durum wheat cultivars. It was suggested by Bordes et al. (2011) that the bread wheat core collection selected from the INRA worldwide wheat core collection (Balfourier et al.,2007), could be a potentially powerful resource to look for genetic associations between markers and nutritional quality traits for human health. As mentioned by Fardet (2010), one of the key issue to improve nutritional benefits of whole-grain wheat products, is to explore the bioavailability of the main bioactive compounds present in wheat grains. Indeed, the bioavailability of "each B vitamin seems to vary greatly" (Fardet, 2010) and "that it is far from 100%". Among the different B vitamins, glycosylated forms of pyridoxine and nicotinic acid are prevalent in plant-derived foods and contribute to human nutrition as partially available sources of pyridoxine and nicotinic acid (Gregory et al., 1991). Simic et al. (2012) have recommended the ratios of mineral elements as bioavailability traits in biofortification research. Such strategy could help to allow the detection of genes that influence the accumulation of bioavailable mineral elements in crop cultivars and thus improve the nutritional density. Therefore, the objectives of the present chapter were to scan the genome for new loci or QTLs involved in the genetic control of wheat vitamins composition as well for bioavailable nicotinic acid and pyridoxine by association mapping in the core collection of 195 accessions studied in chapter 6 and genotyped with diversity array technology (DArT), simple sequence repeats (SSR) and single nucleotide polymorphism (SNP) markers on the wide genome.

2. Results and discussion

2.1. Detection of 370 significant associations

A total of 215 and 155 markers (Table 1; Figure 1) were significantly associated ($p < 0.01$) with B-vitamin and lipide-soluble compound contents respectively. Those markers were detected on all chromosomes (with the largest number of associated marker being on chromosome 5B, 1B, 3B and 7B) in the two environments (Clermont Ferrand 2005-2006, Le Moulon 2006-2007). These numerous markers evidence the complexity of vitamin inheritance (when one marker was associated with several traits, every associations was reported). Chromosomes 3D and 4D were the least informative with only one associated marker. From the 370 associations, only 27 were detected in the two environments, indicating that these markers were relatively stable. Most associations were found for B-vitamins (215 in total), and more precisely for nicotinamide, followed by pantothenic acid and riboflavin. The number of association by location for both group of vitamins were not different. However when looking at individual vitamin trait, locations had a strong impact on the number of associated markers. Indeed, for thiamin, pantothenic acid and riboflavin, 61%, 50% and 56% of the total markers associated with these three traits were respectively found in Clermont-Ferrand. On the contrary, 66%, 68% and 65% of the total

significant markers identified for nicotinamide, pyridoxine and pyridoxal, respectively were found in Le Moulon. For the lipid-soluble compounds, 53% of the total markers associated with lutein were found in Clermont-Ferrand, whereas 58% of the 31 markers associated with β-γ-tocotrienol were found in Le Moulon. Most of the QTLs or AM studies conducted so far focused on grain proteins or carotenoid contents with very little attention given to other grain nutrients. Peleg et al. (2009) studied the genetic and physiological basis of grain protein, micronutrients and macronutrients concentration in tetraploid wheat population of 152 recombinant inbred lines, derived from a cross between durum wheat and wild emmer. They reported that among the eighty-two significant QTLs detected for grain protein and mineral nutrient contents, thirty-eight exhibited GxE interaction. Thus in our study most of the markers associated with vitamins might be significantly affected by association x location interaction.

Table 1. Summary of associations between markers and thirteen vitamin contents detected in the bread wheat core collection grown in 2005-2006 at Clermont-Ferrand, France and in 2006-2007 at Le Moulon, France.

Trait, grain concentration	Markers	Environment		
B-vitamins		All env	Le Moulon	Clermont-Ferrand
Thiamin	26	3	7	16
Nicotinic acid	16	-	7	9
Nicotinamide	41	-	27	14
Pyridoxine	28	-	19	9
Pyridoxal	29	-	19	10
Pantothenic acid	36	6	12	18
Riboflavin	39	2	15	22
lipid-soluble compounds				
α-tocopherol	26	-	14	12
β -γ-tocopherol	22	-	14	8
α-tocotrienol	27	5	9	13
β-γ-tocotrienol	31	1	18	12
β-sitosterol	21	2	10	9
Lutein	28	8	5	15
Total B-vitamins	**215**	**11**	**106**	**98**
Total liposolubles	**155**	**16**	**70**	**69**

2.2. Significant associations detected for each trait

Although, marker-trait associations (MTA) were detected at $p < 0.01$ for all traits, we are reporting only strong MTA ($p < 0.001$) or associations with $p < 0.01$ and r^2values near or > 10%. A summary of MTA in different environments for each phenotypic trait is given in tables 2 and 3.

Considering both criteria (p and r^2), a total of 87 MTA were detected in one or more environments for 13 measured vitamin traits in two environments. Out of these, about 11% of the MTA were detected in two locations and the remaining 89% only in a single environment.

The highest number of MTA was recorded for riboflavin (13) followed by pantothenic acid (11)while the fewest MTA were obtained for nicotinic acid (1), α-tocopherol (3) and β-γ-tocopherol (3).Moreover, pantothenic acid and lutein had the largest number of stable MTA (marker that is associated with a trait in both locations) (4) followed by α-tocotrienol (2).

2.2.1. Thiamin concentrations

Seven markers including one stable markers spread over 6 chromosomes were associated with thiamin contents. The r^2 varied from 8.5% to 14%. The most significant association was with DArT wPt8267 (r^2 of 8.5% and 11% at Clermont-Ferrand and Le Moulon respectively) assigned on chromosome 1BS and 7BL. For DArT wPt 8267, in both locations the individuals carrying the allele wPt 8267 had a significantly ($p<0.05$) higher thiamin content than those not carrying this allele (Supplementary data 4). Synthesis of thiamin in plants involves the independent synthesis of two substituted thiazole and pyrimidine compounds, 4-methyl-5-(2-hydroxyethyl) thiazole phosphate (HET-P) and 4-amino-5-hydroxymethyl-2-methylpyrimidine diphosphate (HMP-PP) which are coupled to form thiamin monophosphate (TMP), the active form of the co-factor. For the building of the thiazole component, plant uses the same pathway developed in yeast, in which NAD^+ and glycine are converted to HET by thiazole synthase (THI4) in cooperation with a sulfur donor (Chatterjee et al., 2007). HET is then phosphorylated to HET-P by the enzyme hydroxyethylthiazole kinase (EC 2.7.1.50). Putative genes coding for hydroxyethylthiazole kinase, were mapped on chromosomes 3B and might be associated with the MTA reported on the same chromosomes and marked by the Gpw7452. The thiamin thiazole synthase putative gene that leads to the biosynthesis of HET was reported on the long arm of chromosome 7B and 7A (Brenchley et al., 2012). It is thus possible that the MTAs detected on 7BL and on 7AL and marked with DArT 8267 and with SSR Barc222 might be associated with this gene. Interestingly, for barc222, the individuals carrying the alleles 194 bp and 202 bp had a significantly higher content of thiamin than those carrying the other three alleles, 200 bp, 204 bp and 206 bp (Supplementary data 4).

2.2.2. Nicotinic acid and nicotinamide concentrations

Only one significant markers (CFA 2297) on chromosome 7AS was associated at Le Moulon with nicotinic acid contents with r^2explaining 15% of the variance. Nine significant markers spread over 8 chromosomes were associated with nicotinamide contents with r^2 ranging between 8% and 21%.The highest percentage of variation explained by a single SSR marker (Barc267 assigned on chromosomes 5BL and 7BS) was 21%. For Barc267, the individuals carrying the allele 171 bp had a significantly higher content of nicotinamide than those carrying the alleles 161 bp, 165 bp, 167 bp, 169bp, 173 bp, 175 bp, 177 bp, 179 bp, 184 bp, 186 bp, and 192 bp (supplementary data 4).The most significant association was with SSR Gpw 4129 marker (r^2 of 14%, $P = 6x10^{-5}$) on chromosome 7DL. Nicotinamide is formed as a catabolite of nicotinamide adenine dinucleotide (NAD) and is a key metabolite of pyridine metabolism (Matsui et al., 2007). One of the enzymes family of the NAD-consuming pathway, is the ADP-ribose transferases (also known as ADP-ribosylation factors (ARF)),

which transfer ADP-ribose from NAD to acceptors with production of nicotinamide. ARF is important for wheat growth and development and it was recently revealed that the ARF expression was affected under different environmental stresses (Pu et al., 2014). The nicotinic acid and nicotinamide MTAs mapped to the chromosomes 1DL, 3BS, 5AL, 5BL, 6AL, 7AS, 7BS and 7DL may reflect variation at ARF genes, since such genes (Brenchley et al., 2012) have been identified on these chromosomes.

2.2.3. Pyridoxal and pyridoxine concentrations

Marker-trait associations were found for pyridoxal contents on chromosomes 3AS, 4AL, 5B, 5DL, 6BL, 7B and 7D, of which the MTAs on chromosomes 6B, 7B and 7D had the strongest r^2values with 24%, 17% and 19% respectively. The most significant MTAs ($p < 0.001$) were detected on chromosome 4A, 5B, 7B and 7D for markers wPt4290, Gpw1164, Gpw1164 and Cfd014 respectively. In Le Moulon, the average pyridoxal contents were highest when the individuals were not carrying the allele wPt 4290 (Supplementary data 4). A total of ten markers spread over seven chromosomes were associated with pyridoxine contents with r^2 ranging between 8% and 25%. The most significant associations were observed on 3BL for SNP marker BPGIPGM.1 ($r^2$of 10%, P = $1x10^{-4}$), on 4AL for DArT wPt6688 (r^2 of 9%, P = $2.67x10^{-4}$) and on 5DL for wPt5505 (r^2 of 8%, P = 6.42x10-4). In plants, two pyridoxal biosynthesis proteins (PDX1/PDX2) directly synthesize pyridoxal-5'-phosphate (PLP) from ribose-5'-phosphate or ribulose-5'-phosphate, in combination with either glyceraldehyde-3'-phosphate or dihydroxyacetone phosphate and glutamine (Mooney et al, 2009). In addition to the direct synthesis of new PLP, the vitamers are interconvertible (pyridoxamine-5'-phosphate (PMP) to PLP or pyridoxine-5'-phosphate (PNP) to PLP) via the so called *salvage pathway.* Besides the biosynthesis of vitamin B6, catabolism of the vitamin is also an important aspect for cellular homeostasis of the compound (Mooney et al, 2009). PLP, PMP and PNP can be dephosphorylated to pyridoxal, pyridoxamine, and pyridoxine, respectively, which can be rephosphorylated by pyridoxal kinase in the cytosols or plastids (Gerdes et al, 2012). Pyridoxal is reduced by a chloroplastic pyridoxal reductase (EC 1.1.1.65) to pyridoxine (Herrero et al, 2011). Putative genes coding for pyridoxal biosynthesis, mapped on chromosomes 4AL (Brenchley et al., 2012) might be associated with the MTA reported on the same chromosome and marked by the DArT wPt4290. Pyridoxine-5'-phosphate oxidase (EC 1.4.3.5) is an enzyme that catalyzes the biosynthesis of pyridoxal-5'-posphate from either pyridoxine-5'-phosphate or pyridoxamine-5'-phosphate. The strong and significant MTAs associated with pyridoxal and pyridoxine contents detected on the long arm of chromosome 6B and on the short arm of chromosome 2D, 7B and 7D may reflect variation at pyridoxine-5'-phosphate oxidase genes , since putative genes associated with this enzyme has been mapped on chromosome 2DS,6BL, 7BS and 7DS (Brenchley et al., 2012). Pyridoxal phosphate phosphatase (EC 3.1.3.74) catalyzes not only the dephophorylation of PLP but also the dephosphorylation of PNP and PMP. Putative genes associated with pyridoxal phosphate phosphatase in the vitamin B6 pathway, was mapped on chromosome 3BS and on chromosome 7DL (Brenchley et al., 2012). It is therefore possible that the significant

MTAs found for pyridoxal and pyridoxine contents on chromosome 3BS and 7DL might be due to variation at pyridoxal phosphate phosphatase genes.

2.2.4. Riboflavin concentrations

The most stable MTA for riboflavin contents was detected on chromosome 7DL for markers Gpw 350 with r^2 of 23% and 21% at Clermont-Ferrand and Le Moulon respectively. For Gpw 350, the individuals carrying the allele 218 bp had a significantly higher content of riboflavin than those carrying the alleles, 191 bp, 224 bp, 225 bp and 228 bp (Supplementary data 4). The highest r^2 values (45% at Clermont-Ferrand with P = $1.21x10^{-7}$) was observed on 2DS for SSR marker Gwm 261. Out of the 13 markers recorded for this trait, 12 were strongly associated with $p < 0.001$. Riboflavin synthase (EC 2.5.1.9) is an enzyme that catalyzes the final reaction of riboflavin biosynthesis. The MTA (wPt5914) found for riboflavin contents and mapped on the chromosome 5BS may reflect variation at riboflavin synthase gene, since such gene has been identified on this chromosome (Brenchley et al., 2012). Riboflavin biosynthesis protein (RibBA) (EC.4.1.99.12), catalyzes the conversion of D-ribulose 5-phosphate to formate and 3,4-dihydroxy-2-butanone 4-phosphate (Jia et al., 2013). Physical location of genes coding for RibBA in common wheat has been reported to be on chromosomes 6AL, 6DL, 7AL, 7BL and 7DL (Brenchley et al., 2012). The presence of significant MTA for riboflavin contents on these chromosomes may be due to association with genes coding for RibBA.

2.2.5. Pantothenic acid concentrations

The MTAs for pantothenic acid content were distributed on chromosomes 1B, 3AS, 3B, 5DS, 7A and 7BS. The most significant associations were with markers Wms282 (r^2 of 41%, P = $8x10^{-3}$), Wms332 (r^2 of 42%, P = $4x10^{-3}$), CFA2297 (r^2 of 24%, P = $4.41x10^{-5}$) on chromosome 7A, the marker Barc147 (r^2 of 19%, P = $2.4x10^{-4}$), Gpw 7452 (r^2 of 15%, P = $6.4x10^{-4}$)on chromosome 3B and the SSR marker Gwm190 (r^2 of 26%, P = $1.7x10^{-4}$) on chromosome 5D. For Gpw 7452, at both sites, the average pantothenic acid contents were highest when the individuals were carrying the allele 137 bp (Supplementary data 4). For Wms282, the individuals carrying the allele 220 bp had a significantly higher content of pantothenic acid than those carrying the alleles, 204 bp, 210 bp, 212 bp, 214 bp, 216 bp, 218 bp, 224 bp, 239 bp, 249 bp, 251 bp and 284 bp. Whereas, for Wms332, the individuals carrying the allele 234 bp had a significantly higher content of pantothenic acid than those carrying the alleles, 224 bp, 226 bp, 228 bp, 230 bp, 232 bp, 238 bp, 253 bp, 263 bp, 265 bp and 298 bp (Supplementary data 4). The genes coding for the enzyme dephospho-CoA (EC 2.7.1.24) kinase and for phosphopantothenate-cysteine kinase (EC 6.3.2.5)that leads to the biosynthesis of coenzyme A have been reported on chromosome 3B, 6BL,6DL and 7AL (Choulet et al., 2014; Brenchley et al., 2012; Choulet et al., 2010). In our study, strong associations for pantothenic contents were detected on chromosome 3B and on the long arm of chromosome 7A. Thus quantitative variations observed for this trait could be related to the presence of these genes. Falk and Guerra (1993) reported that the first committed step toward coenzyme A synthesis was catalyzed by pantothenate kinase (EC 2.7.1.33).

Putative genes coding for pantothenate kinase were mapped on chromosomes 7AS and 7BS (Brenchley et al., 2012) and might be associated with the two MTAs found for pantothenic acid contents on these chromosomes (CFA2297 and Gwm333). Interestingly, for Gwm333 at Clermont-Ferrand the individuals carrying the allele 169 bp had a significantly ($p<0.05$) higher pantothenic acid contents than those carrying the allele 167 bp (Supplementary data 4).

2.2.6. Vitamin E concentrations

All MTAs detected for α-tocopherol, β -γ-tocopherol and β-γ-tocotrienol contents on chromosomes 3AL, 3BL, 4A, 4BL, 6AL,7AS and 7DS were found only at Le Moulon with the presence of very strong associations for some MTA (r^2 varied from 10% to 21%). The most stable MTA for β-γ-tocotrienol contents was detected on chromosome 2BL with DArT wPt 8460 with r^2 of 6% and 8% at Clermont-Ferrand and Le Moulon respectively, table 3. For DArT wPt 8460, in both sites, the average β-γ-tocotrienol contents were highest when the individuals were not carrying the allele wPt 8460 (Supplementary data 4) Four markers including two stable markers were significantly associated with α-tocotrienol contents. The highest r^2 values (r^2 of 15%, $P = 7x10^{-3}$) were observed on chromosome 5DL for SSR marker Gwm272. The plant tocopherol pathway utilizes cytosolic aromatic amino acid metabolism for synthesis of the chromanol group (homogentisic acid-HGA) and the plastidic deoxyxylulose 5-phospate pathway for synthesis of the side chain (phytyl-diphosphate phytilDP for tocopherols and geranyl-geranyl diphosphate GDDP for tocotrienols). The first step in tocopherol synthesis involves the production of the aromatic head group, HGA from p-hydroxyphenylpyruvic acid (HPP) by the enzyme p-hydroxyphenylpyruvic acid dioxygenase (HPPD, EC 1.13.11.27). HGA is then subject to prenylation with phytyl-diphosphate or geranyl-geranyl diphosphate to yield 2-methyl-6-phytylplastoquinol (MPBQ) and 2-methyl-6-geranylgeranylplastoquinol (MGGBQ), respectively, by the enzyme homogentisate prenyltransferase (HPT, EC 2.5.1.115) for MPBQ or by the enzyme homgentisate geranylgeranyl transferase(HGGT; EC 2.5.1.116) for MGGBQ. MPBQ and MGGBQ are the first committed intermediates in the synthesis of all tocopherols and tocotrienols, respectively. The substrate specificity of HPT is a key factor determining whether tocopherols, tocotrienols or both compounds can be made in an organism. The next steps in synthesis are ring methylations (EC 2.1.195) and/or ring cyclization (EC 5.5.1.24) (Dellapenna, 2005). Physical location of genes coding for homogentisate 1-2 dioxygenase (EC 1.13.11.5), homogentisate prenyltransferase and gama-tocopherol methyltransferase (EC 2.1.1.95) in common wheat have been reported to be on chromosomes 7AS, 7DS, 7AL and 6AL respectively (Brenchley et al., 2012). The presence of significant MTA for vitamin contents on these chromosomes may be due to association with genes coding for these enzymes.

2.2.7. β-sitosterol concentrations

A total of seven MTAs were obtained for β-sitosterol contents on chromosomes 1BS, 2BS, 3BL, 5AS, 6AL and 7AS. The strongest MTA (r^2 of 15%) were detected on chromosome 3BL

for Barc77 at Le Moulon and on chromosome 6AL for SSR marker Gwm642 at Clermont-Ferrand. For barc77, the individuals carrying the allele 166 bp had a significantly ($p<0.05$) higher β-sitosterol contents than those carrying the allele 182bp, 186 bp, 230 bp and 234 bp (Supplementary data 4).Putative genes coding for the three enzymes cycloartenol synthase (EC 5.4.99.8), cycloeucalenol cycloisomerase (EC 5.5.1.9) and methylsterol monooxygenase (EC 1.14.13.72), which participates in biosynthesis of sterols have been respectively mapped on chromosomes 5AS, 6AL and 7AS (Brenchley et al., 2012). These putative genes might be associated with markers Gwm415, Gwm642 and Barc222 reported on the same chromosomes and strongly associated with sterol contents. Interestingly, for Gwm415 the individuals carrying the allele 135 bp had a significantly ($p<0.05$) higher β-sitosterol contents than those carrying the allele 129 bp (Supplementary data 4).

2.2.8. Carotenoids concentrations

The three stable associated markers were spread over three chromosomes (2BS, 5AS and 6AS). The strongest stable association was with SSR marker Gwm257 (r^2 of 14% and 18% Clermont-Ferrand and Le Moulon, respectively) on chromosome 2BS. Biosynthesis of all carotenoids starts from phytoene and is catalysed by a relatively low number of enzymes (Cunningham and Gantt, 1998). Putative genes coding for geranyl-geranyl pyrosphosphate synthase (EC 2.5.1.29), an enzyme important for the biosynthesis of lutein, were mapped on the homologeous chromosomes of group 2 (Cenci et al., 2010), and this putative gene might be associated with the marker Gwm257 on chromosome 2BS detected in the present study and significantly associated with lutein contents. The minor MTAs on chromosome 3BL and 3BS (Figure 1) have also been described. Howitt et al. (2009) identified the candidate ε-cyclase gene that leads to the biosynthesis of lutein, and showed the association of this gene with the QTL on the proximal region of 3BS. Furthermore, Blanco et al. (2011) reported QTL associated with lutein concentration on the long arm of chromosome 3B. The stable MTA detected in the present study on the short arm of chromosome 5A, marked by the SSR Gwm415, appears to be at a similar position than the 5A QTL identified by Blanco et al. (2011) in durum wheat. Reimer et al. (2008) reported on 6AS a significant association between marker barc 146 and yellow pigment in durum wheat. The stable SSR (WPt8266) associated with lutein contents and located on chromosome 6AS was also identified in our study. Putative genes coding for the enzyme lycopene betacyclase (EC 5.5.1.19) and geranylgeranyl pyrophosphate synthase which both participate in biosynthesis of lutein have been mapped on chromosome 6AS (Brenchley et al., 2012). These putative genes might be associated with markers WPt8266. In addition, as described by Reimer et al. (2008) several marker clusters ($p <0.01$) were identified on chromosome 1B. Interestingly, the importance of the group of chromosome 7 (Pozniak et al., 2007) to yellow pigment expression in durum wheat was not confirmed in this study, as only one significant association on the distal end of chromosome 7DL was identified. To summarize, in this study most of the markers identified were in chromosomal regions where QTL for carotenoid concentration have been reported.

Table 2. Strongest R^2 and significance of association between markers and B-vitamin contents at Clermont-Ferrand and Le Moulon.

Chromosome	Marker	Allele	Clermont-Ferrand		Allele	Le Moulon	
Thiamin							
1B	**wPt8267**	**1**	**8.5**	**	**1**	**11**	**
3A	Platz4-A.3				G	9	**
3B	Gpw7452		12	*			
4A	Cfa2140		12	*			
7A	Barc222	202;194	11	*			
7B	Gpw3111	191	13	*			
	Gwm333	165	14	*			
	wPt8267	**1**	**8.5**	**	**1**	**11**	**
Nicotinic acid							
7A	CFA2297					15	*
Nicotinamide							
1D	PGKD.2				A	9	**
3B	wPt6973	1	9	**			
5A	wPt0373	0	8	**			
5B	Barc267	171	21	*			
6A	Gpw7384				227	8	**
7A	CFA2297					14	*
7B	Barc267	171	21	*			
	Gwm333					12	*
7D	Gpw4129		14	**			

Table 2. (Continued)

Chromosome	Marker	Allele	Clermont-Ferrand		Allele	Le Moulon	
Pyridoxal							
3A	LEC1-A					10	*
4A	wPt4290				0	8	**
5B	Gpw1164		10	**			
5D	Cfd8					16	*
6B	Gwm219					24	*
7B	Gpw4432					17	*
	Gpw1164		10	**			
	Barc176					17	*
7D	Cfd014					14	**
	Gpw5164		19	*			
Pyridoxine							
1A	Gwm135					25	*
2D	Gwm261					22	*
3A	LEC1-A					10	*
3B	Barc147	125	13	*			
	HGA1start-3B					9	**
	Gpw7452	137	12	*			
	BPGIPGM.1					10	**
4A	wPt6688					9	**
5D	wPt5505					8	**
7D	Cfd014					11	*

Table 2. (Continued)

Chromosome	Marker	Allele	Clermont-Ferrand		Allele	Le Moulon	
Riboflavin							
1B	wPt1911		9	**			
2D	Gwm261		45	**			
3A	wPt9154				1	7	**
5B	wPt5914					10	*
	wPt2707		15	**			
6A	wPt8006	0	8	**			
	wPt9976	0	8	**			
6B	wPt5333	1	7	**			
6D	Gpw7433				337	12	**
7A	wPt4038					8	**
7B	Barc176		25	**			
	wPt4038					8	**
7D	**Gpw350**		**23**	**		**21**	*
Pantothenic acid							
1B	wPt6012					9	**
	GluB1.1	G	6	**	G	5	*
3A	wPt6012					9	**
3B	Barc147		19	**			
	Gpw7452	**137**	**15**	**	**137**	**11**	*
	Barc164		**22**	*		**21**	*
5D	Gwm190		26	**			
7A	CFA2297		24	**			
	Wms332	234	42	*			
	Wms282	220	41	*			
7B	**Gwm333**	**169**	**24**	**		**14**	*

*0.001 < p value < 0.01, **p value < 0.001. In bold, markers significantly associated for the two sites. Note: Alleles of the markers which lead to a significantly higher content of vitamins (associated with those markers) were reported on table 2 (from supplementary data 4)

Table 3. Strongest R^2 and significance of association between markers and lipide-soluble compound contents at Clermont-Ferrand and Le Moulon.

Chromosome	Marker	Allele	Clermont-Ferrand		Allele	Le Moulon	
α-tocopherol							
4A	CFA2140					11	*
7A	CFA2264					15	*
7D	cfd066					21	*
β -γ-tocopherol							
3A	Platz4-A.2				A	11	**
3B	39310-3B				C	10	**
6A	Gwm427					20	*
7D	Cfd066					21	*
α-tocotrienol							
3A	Vip-3A					8	**
5A	**Gwm415**		**11**	*		**12**	*
5D	Gwm272		15	*			
7B	**SAL1-B.3**	**C**	**6**	**	**T**	**6**	**
β-γ-tocotrienol							
2B	**wPt8460**	**0**	**6**	*	**0**	**8**	**
4A	Gpw5102					13	*
	Gwm160				207	10	*
4B	Gwm251					23	*
7D	wPt6117				0	8	**
	Gpw5102					13	*

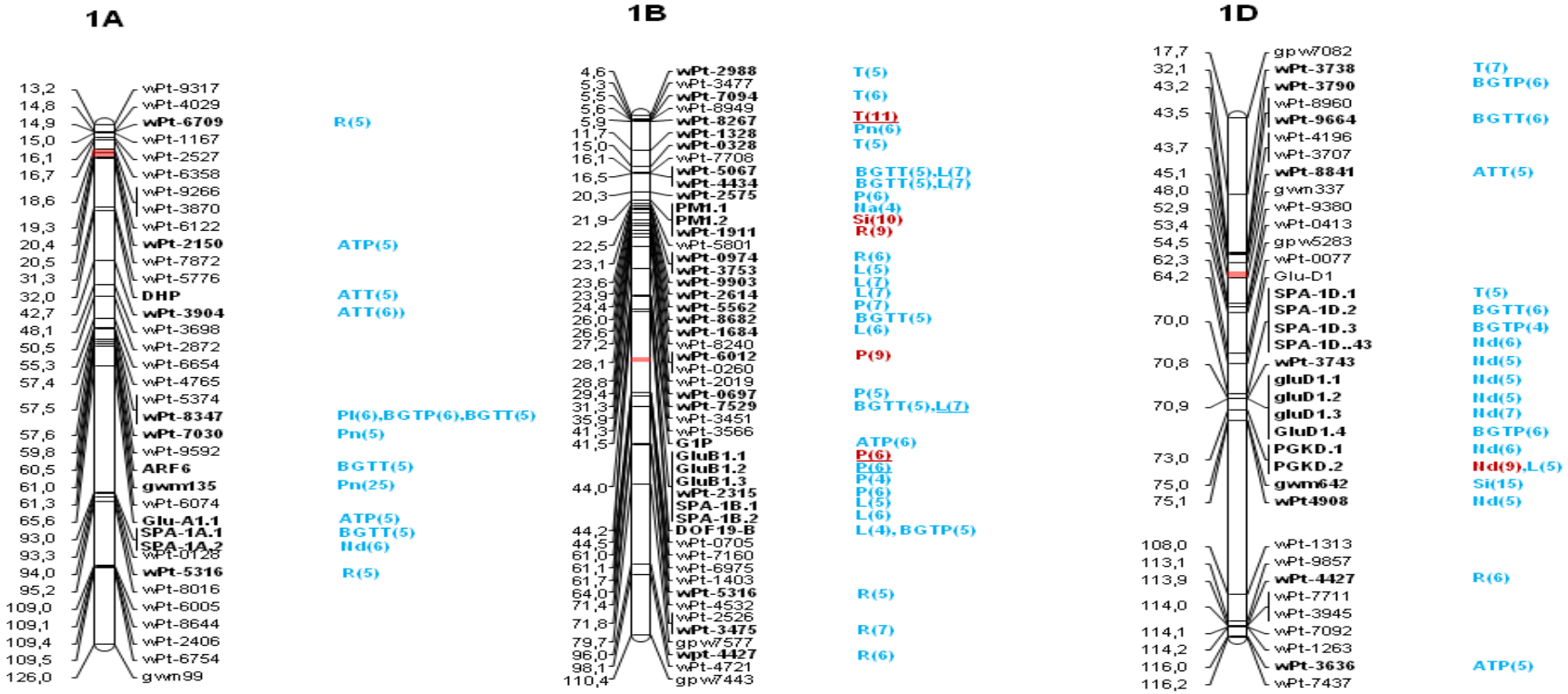
Table 3. (Continued)

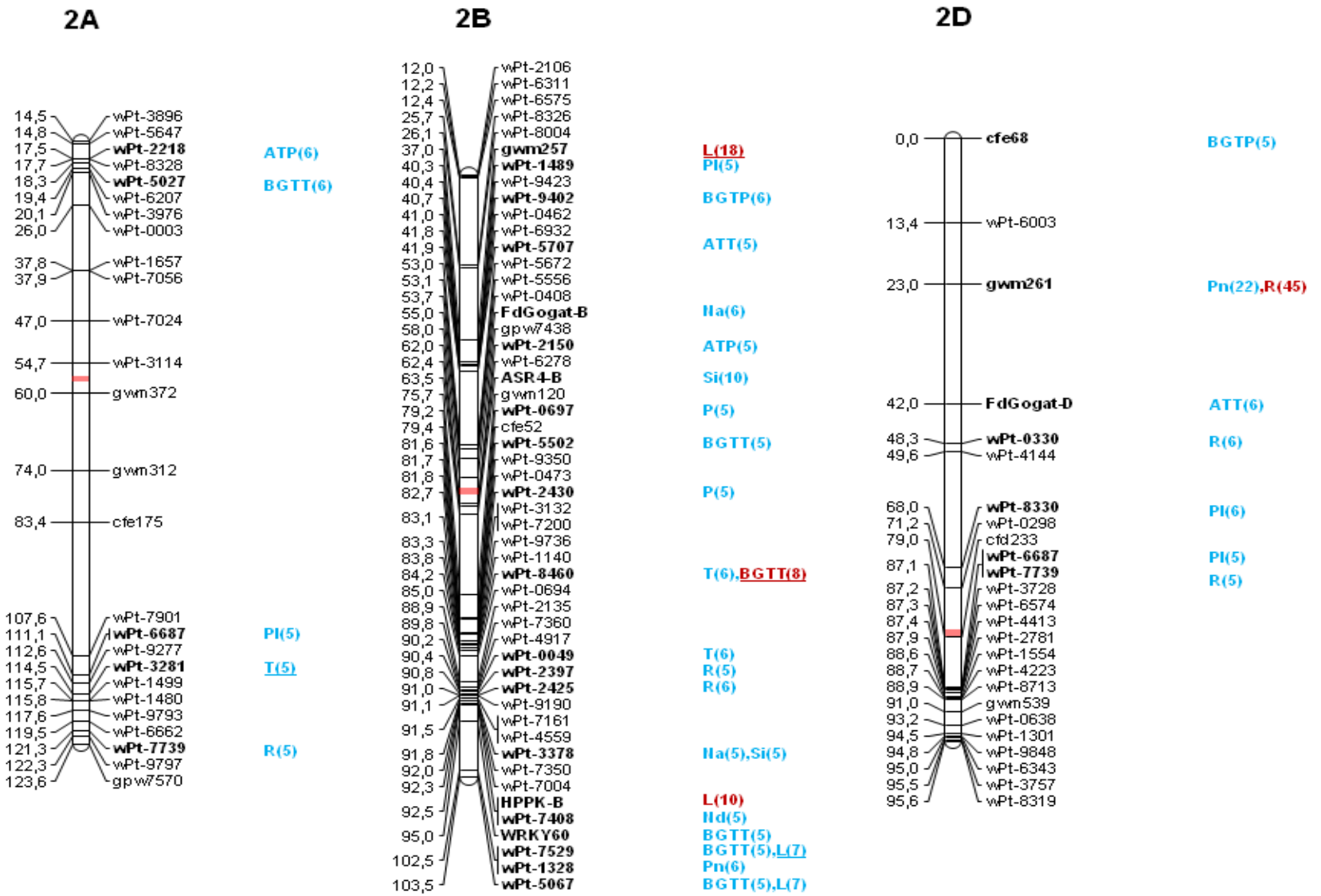
β-sitosterol	Marker	Allele	Clermont-Ferrand		Allele	Le Moulon	
1B	PM1.2	C	10	**			
2B	ASR4-B		10	**			
3B	LDD-B					8	**
	Barc77				166	15	*
5A	Gwm415				135	11	*
6A	Gwm642		15	*			
7A	Barc222		14	*			
Lutein							
2B	**Gwm257**		**14**	*		**18**	**
	HPPK-B	A	10	**			
5A	**Gwm415**		**13**	**		**10**	*
6A	**WPt8266**		**10**	**		**9**	**

*0.001 < p value < 0.01, **p value < 0.001. In bold, markers significantly associated for the two sites.

Note: Alleles of the markers which lead to a significantly higher content of compound (associated with those markers) were reported on table 3 (from supplementary data 4)

Figure 1: Significant marker-trait associations for T(thiamine),Pl(Pyridoxal),Pn(Pyridoxine),Na(nicotinic acid), Nd(Nicotinamide), R(Riboflavin), P(Pantothenic acid), ATP(α-Tocopherol), BGTP(β -γ-tocopherol),ATT(α -Tocotrienol), BGTT(β- γ-Tocotrienol), L(Lutein), Si(Beta-Sitosterol). Markers in bold were significant. Numbers in parentheses refer to the variability explained by the marker as a percentage of the total variability. Traits in blue and red are associations which were considered as significant at p≤0,01 and p ≤ 0,001 respectively. Traits with an underline are associations which were considered as significant in all two testing environments. Approximate position of the centromere is marked with. For clarity, in high-marker density regions some markers are not shown on the map

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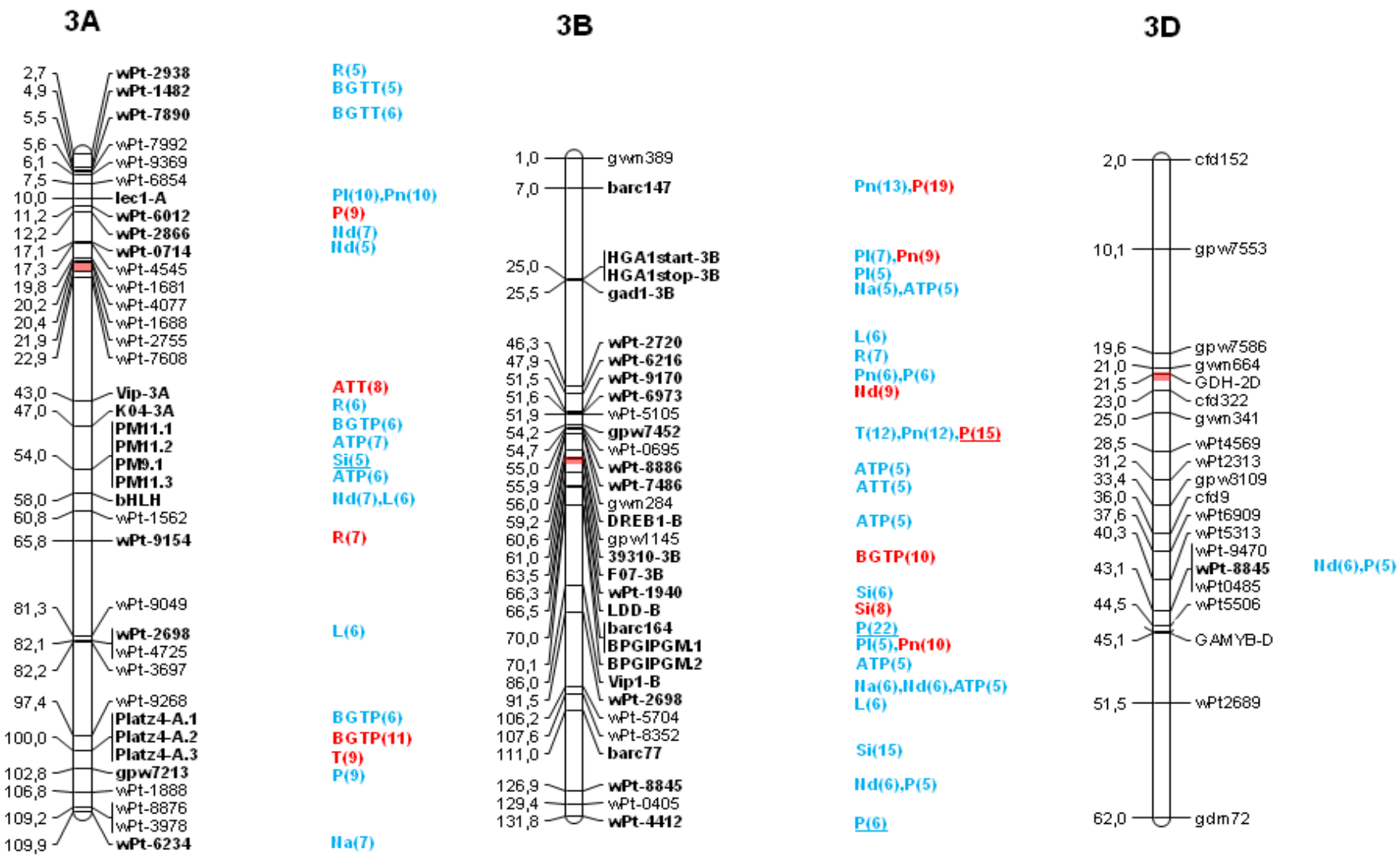
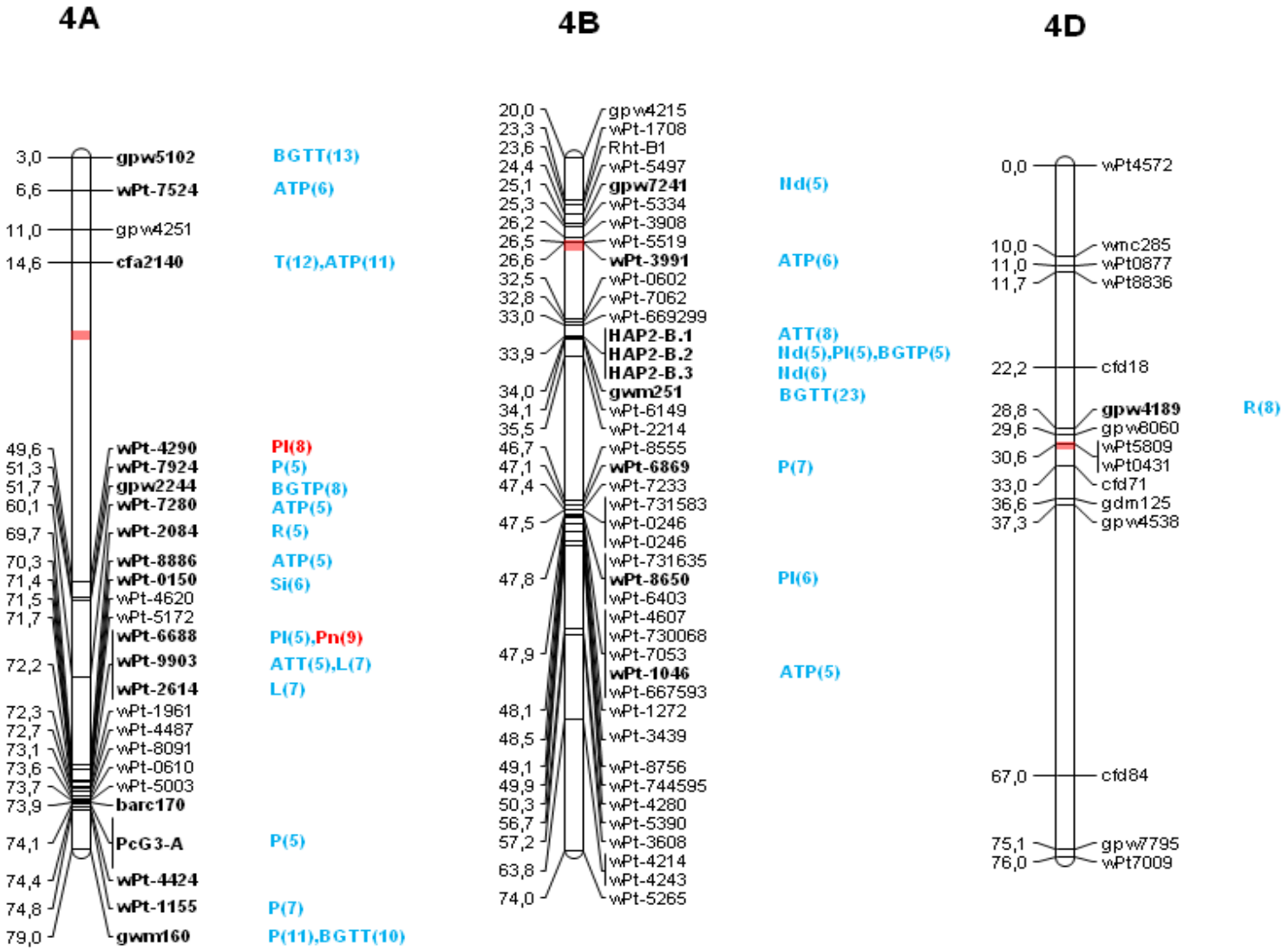
14,5 wPt-3896
14,8 wPt-5647
17,5 wPt-2218
17,7 wPt-8328
18,3 wPt-5027
19,4 wPt-6207
20,1 wPt-3976
26,0 wPt-0003
37,8 wPt-1657
37,9 wPt-7056
47,0 wPt-7024
54,7 wPt-3114
60,0 gwm372
74,0 gwm312
83,4 cfe175
107,6 wPt-7901
111,1 wPt-6687
112,6 wPt-9277
114,5 wPt-3281
115,7 wPt-1499
115,8 wPt-1480
117,6 wPt-9793
119,5 wPt-6662
121,3 wPt-7739
122,3 wPt-9797
123,6 gpw7570
ATP(6)
BGTT(6)
Pl(5)
T(5)
R(5)
2B
12,0 wPt-2106
12,2 wPt-6311
12,4 wPt-6575
25,7 wPt-8326
26,1 wPt-8004
37,0 gwm257
40,3 wPt-1489
40,4 wPt-9423
40,7 wPt-9402
41,0 wPt-0462
41,8 wPt-6932
41,9 wPt-5707
53,0 wPt-5672
53,1 wPt-5556
53,7 wPt-0408
55,0 FdGogat-B
58,0 gpw7438
62,0 wPt-2150
62,4 wPt-6278
63,5 ASR4-B
75,7 gwm120
79,2 wPt-0697
79,4 cfe52
81,6 wPt-5502
81,7 wPt-9350
81,8 wPt-0473
82,7 wPt-2430
83,1 wPt-3132
wPt-7200
83,3 wPt-9736
83,8 wPt-1140
84,2 wPt-8460
85,0 wPt-0694
88,9 wPt-2135
89,8 wPt-7360
90,2 wPt-4917
90,4 wPt-0049
90,8 wPt-2397
91,0 wPt-2425
91,1 wPt-9190
wPt-7161
91,5 wPt-4559
91,8 wPt-3378
92,0 wPt-7350
92,3 wPt-7004
92,5 HPPK-B
wPt-7408
95,0 WRKY60
102,5 wPt-7529
wPt-1328
103,5 wPt-5067
L(18)
Pl(5)
BGTP(6)
ATT(5)
Na(6)
ATP(5)
Si(10)
P(5)
BGTT(5)
P(5)
T(6),BGTT(8)
T(6)
R(5)
R(6)
Na(5),Si(5)
L(10)
Nd(5)
BGTT(5)
BGTT(5),L(7)
Pn(6)
BGTT(5),L(7)
2D
0,0 cfe68
13,4 wPt-6003
23,0 gwm261
42,0 FdGogat-D
48,3 wPt-0330
49,6 wPt-4144
68,0 wPt-8330
71,2 wPt-0298
79,0 cfd233
87,1 wPt-6687
wPt-7739
87,2 wPt-3728
87,3 wPt-6574
87,4 wPt-4413
87,9 wPt-2781
88,6 wPt-1554
88,7 wPt-4223
88,9 wPt-8713
91,0 gwm539
93,2 wPt-0638
94,5 wPt-1301
94,8 wPt-9848
95,0 wPt-6343
95,5 wPt-3757
95,6 wPt-8319
BGTP(5)
Pn(22),R(45)
ATT(6)
R(6)
Pl(6)
Pl(5)
R(5)


3A
2,7 wPt-2938
4,9 wPt-1482
5,5 wPt-7890
5,6 wPt-7992
6,1 wPt-9369
7,5 wPt-6854
10,0 Iec1-A
11,2 wPt-6012
12,2 wPt-2866
17,1 wPt-0714
17,3 wPt-4545
19,8 wPt-1681
20,2 wPt-4077
20,4 wPt-1688
21,9 wPt-2755
22,9 wPt-7608
43,0 Vip-3A
47,0 K04-3A
54,0 PM1.1 PM1.2 PM9.1 PM1.3
58,0 bHLH
60,8 wPt-1562
65,8 wPt-9154
81,3 wPt-9049
82,1 wPt-2698
82,2 wPt-4725 wPt-3697
97,4 wPt-9268
100,0 Platz4-A.1 Platz4-A.2 Platz4-A.3
102,8 gpw7213
106,8 wPt-1888
109,2 wPt-8876 wPt-3978
109,9 wPt-6234
R(5)
BGTT(5)
BGTT(6)
Pl(10),Pn(10)
P(9)
Nd(7)
Nd(5)
ATT(8)
R(6)
BGTP(6)
ATP(7)
Si(5)
ATP(6)
Nd(7),L(6)
R(7)
L(6)
BGTP(6)
BGTP(11)
T(9)
P(9)
Na(7)
3B
1,0 gwm389
7,0 barc147
25,0 HGA1start-3B HGA1stop-3B
25,5 gad1-3B
46,3 wPt-2720
47,9 wPt-6216
51,5 wPt-9170
51,6 wPt-6973
51,9 wPt-5105
54,2 gpw7452
54,7 wPt-0695
55,0 wPt-8886
55,9 wPt-7486
56,0 gwm284
59,2 DREB1-B
60,6 gpw1145
61,0 39310-3B
63,5 F07-3B
66,3 wPt-1940
66,5 LDD-B
70,0 barc164 BPGIPGM1 BPGIPGM2
70,1 Vip1-B
86,0 wPt-2698
91,5 wPt-5704
106,2 wPt-8352
107,6 barc77
111,0
126,9 wPt-8845
129,4 wPt-0405
131,8 wPt-4412
Pn(13),P(19)
Pl(7),Pn(9)
Pl(5)
Na(5),ATP(5)
L(6)
R(7)
Pn(6),P(6)
Nd(9)
T(12),Pn(12),P(15)
ATP(5)
ATT(5)
ATP(5)
BGTP(10)
Si(6)
Si(8)
P(22)
Pl(5),Pn(10)
ATP(5)
Na(6),Nd(6),ATP(5)
L(6)
Si(15)
Nd(6),P(5)
P(6)
3D
2,0 cfd152
10,1 gpw7553
19,6 gpw7586
21,0 gwm664
21,5 GDH-2D
23,0 cfd322
25,0 gwm341
28,5 wPt4569
31,2 wPt2313
33,4 gpw3109
36,0 cfd9
37,6 wPt6909
40,3 wPt5313
43,1 wPt-9470 wPt-8845 wPt0485
44,5 wPt5506
45,1 GAMYB-D
51,5 wPt2689
62,0 gdm72
Nd(6),P(5)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3,0 gpw5102 BGTT(13)
6,6 wPt-7524 ATP(6)
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14,6 cfa2140 T(12),ATP(11)
49,6 wPt-4290 Pl(8)
51,3 wPt-7924 P(5)
51,7 gpw2244 BGTP(8)
60,1 wPt-7280 ATP(5)
69,7 wPt-2084 R(5)
70,3 wPt-8886 ATP(5)
71,4 wPt-0150 Si(6)
71,5 wPt-4620
71,7 wPt-5172
wPt-6688 Pl(5),Pn(9)
72,2 wPt-9903 ATT(5),L(7)
wPt-2614 L(7)
72,3 wPt-1961
72,7 wPt-4487
73,1 wPt-8091
73,6 wPt-0610
73,7 wPt-5003
73,9 barc170
74,1 PcG3-A P(5)
74,4 wPt-4424
74,8 wPt-1155 P(7)
79,0 gwm160 P(11),BGTT(10)
4B
20,0 gpw4215
23,3 wPt-1708
23,6 Rht-B1
24,4 wPt-5497
25,1 gpw7241 Nd(5)
25,3 wPt-5334
26,2 wPt-3908
26,5 wPt-5519
26,6 wPt-3991 ATP(6)
32,5 wPt-0602
32,8 wPt-7062
33,0 wPt-669299
HAP2-B.1 ATT(8)
33,9 HAP2-B.2 Nd(5),Pl(5),BGTP(5)
HAP2-B.3 Nd(6)
34,0 gwm251 BGTT(23)
34,1 wPt-6149
35,5 wPt-2214
46,7 wPt-8555
47,1 wPt-6869 P(7)
47,4 wPt-7233
wPt-731583
47,5 wPt-0246
wPt-0246
wPt-731635
47,8 wPt-8650 Pl(6)
wPt-6403
wPt-4607
wPt-730068
47,9 wPt-7053
wPt-1046 ATP(5)
wPt-667593
48,1 wPt-1272
48,5 wPt-3439
49,1 wPt-8756
49,9 wPt-744595
50,3 wPt-4280
56,7 wPt-5390
57,2 wPt-3608
63,8 wPt-4214
wPt-4243
74,0 wPt-5265
4D
0,0 wPt4572
10,0 wmc285
11,0 wPt0877
11,7 wPt8836
22,2 cfd18
28,8 gpw4189 R(8)
29,6 gpw8060
30,6 wPt5809
wPt0431
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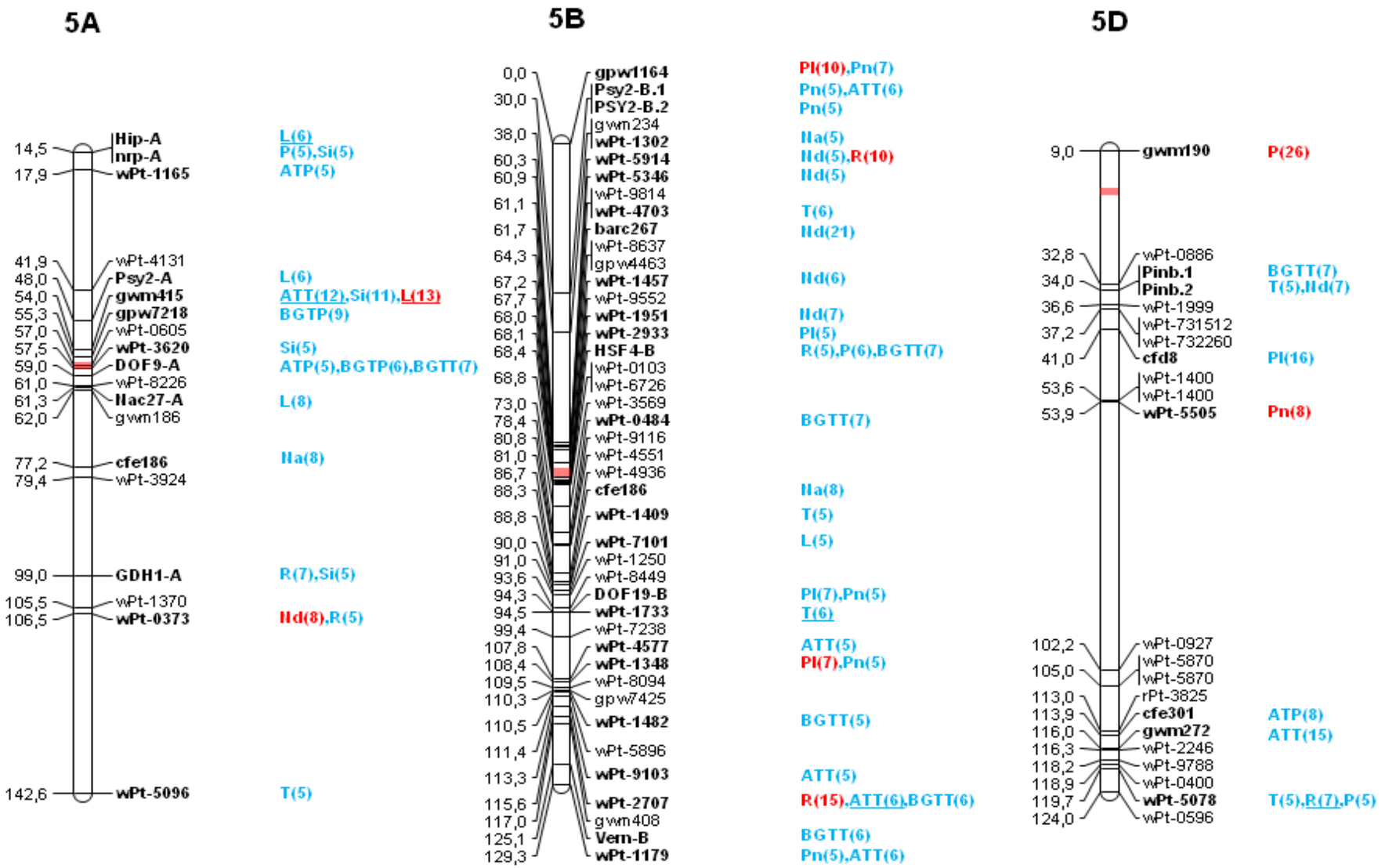
36,6 gdm125
37,3 gpw4538
67,0 cfd84
75,1 gpw7795
76,0 wPt7009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nrp-A
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57,0 wPt-0605
57,5 wPt-3620
59,0 DOF9-A
61,0 wPt-8226
61,3 Nac27-A
62,0 gwm186
77,2 cfe186
79,4 wPt-3924
99,0 GDH1-A
105,5 wPt-1370
106,5 wPt-0373
142,6 wPt-5096
L(6)
P(5),Si(5)
ATP(5)
L(6)
ATT(12),Si(11),L(13)
BGTP(9)
Si(5)
ATP(5),BGTP(6),BGTT(7)
L(8)
Na(8)
R(7),Si(5)
Nd(8),R(5)
T(5)
5B
0,0 gpw1164
30,0 Psy2-B.1
PSY2-B.2
38,0 gwm234
60,3 wPt-1302
60,9 wPt-5914
61,1 wPt-5346
61,7 wPt-9814
64,3 wPt-4703
67,2 barc267
67,7 wPt-8637
68,0 gpw4463
68,1 wPt-1457
68,4 wPt-9552
68,8 wPt-1951
73,0 wPt-2933
78,4 HSF4-B
80,8 wPt-0103
81,0 wPt-6726
86,7 wPt-3569
88,3 wPt-0484
88,8 wPt-9116
90,0 wPt-4551
91,0 wPt-4936
93,6 cfe186
94,3 wPt-1409
94,5 wPt-7101
99,4 wPt-1250
107,8 wPt-8449
108,4 DOF19-B
109,5 wPt-1733
110,3 wPt-7238
110,5 wPt-4577
111,4 wPt-1348
113,3 wPt-8094
115,6 gpw7425
117,0 wPt-1482
125,1 wPt-5896
129,3 wPt-9103
wPt-2707
gwm408
Vern-B
wPt-1179
Pl(10),Pn(7)
Pn(5),ATT(6)
Pn(5)
Na(5)
Nd(5),R(10)
Nd(5)
T(6)
Nd(21)
Nd(6)
Nd(7)
Pl(5)
R(5),P(6),BGTT(7)
BGTT(7)
Na(8)
T(5)
L(5)
Pl(7),Pn(5)
T(6)
ATT(5)
Pl(7),Pn(5)
BGTT(5)
ATT(5)
R(15),ATT(6),BGTT(6)
BGTT(6)
Pn(5),ATT(6)
5D
9,0 gwm190
32,8 wPt-0886
34,0 Pinb.1
Pinb.2
36,6 wPt-1999
37,2 wPt-731512
41,0 wPt-732260
cfd8
53,6 wPt-1400
53,9 wPt-1400
wPt-5505
102,2 wPt-0927
105,0 wPt-5870
wPt-5870
113,0 rPt-3825
113,9 cfe301
116,0 gwm272
116,3 wPt-2246
118,2 wPt-9788
118,9 wPt-0400
119,7 wPt-5078
124,0 wPt-0596
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T(5),Nd(7)
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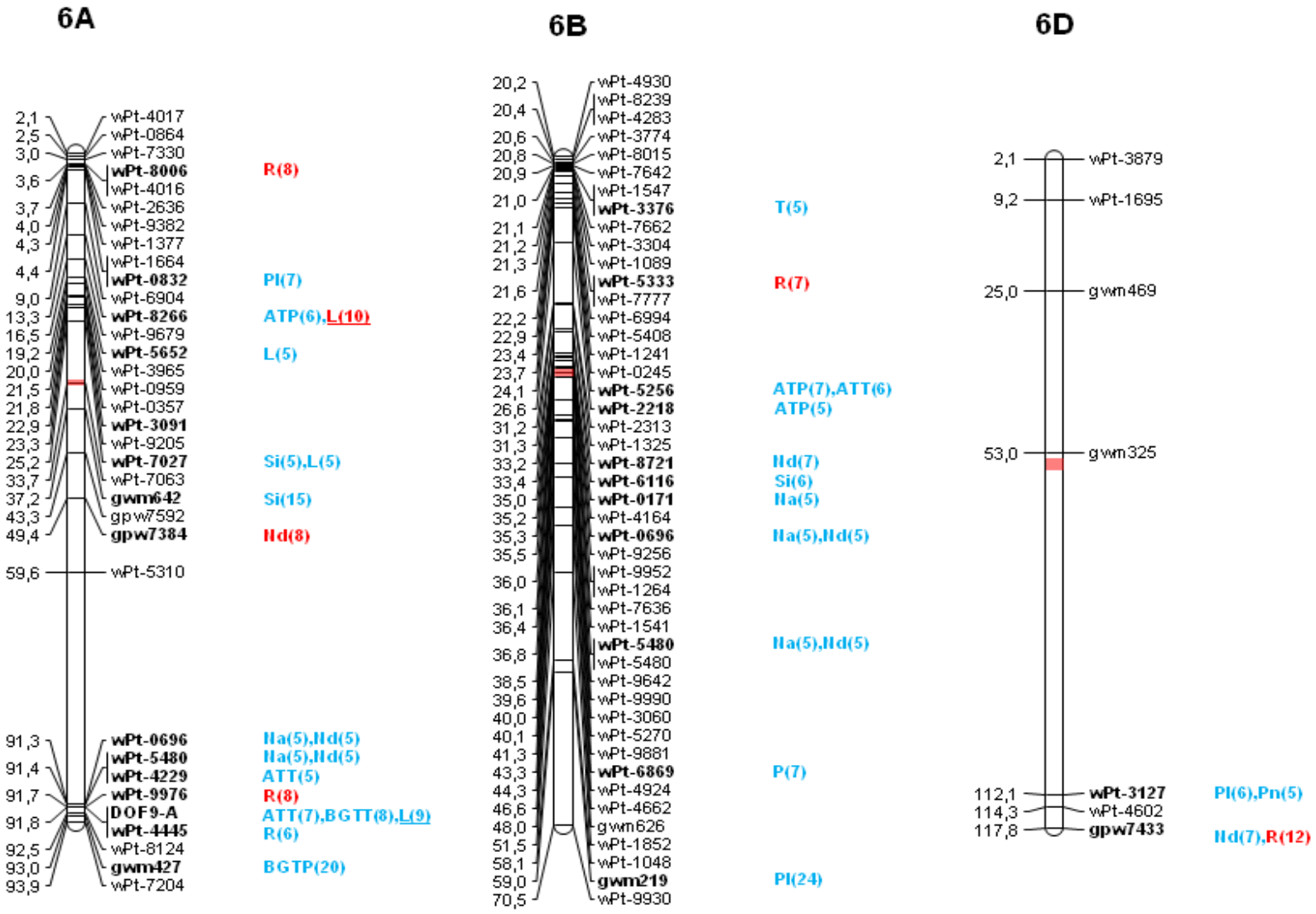
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wPt-8006
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4,3 wPt-1377
wPt-1664
4,4 wPt-0832
9,0 wPt-6904
13,3 wPt-8266
16,5 wPt-9679
19,2 wPt-5652
20,0 wPt-3965
21,5 wPt-0959
21,8 wPt-0357
22,9 wPt-3091
23,3 wPt-9205
25,2 wPt-7027
33,7 wPt-7063
37,2 gwm642
43,3 gpw7592
49,4 gpw7384
59,6 wPt-5310
91,3 wPt-0696
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wPt-4229
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DOF9-A
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R(6)
BGTP(20)
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wPt-4283
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wPt-1547
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21,3 wPt-1089
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24,1 wPt-5256
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R(7)
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Nd(7)
Si(6)
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P(7)
Pl(24)
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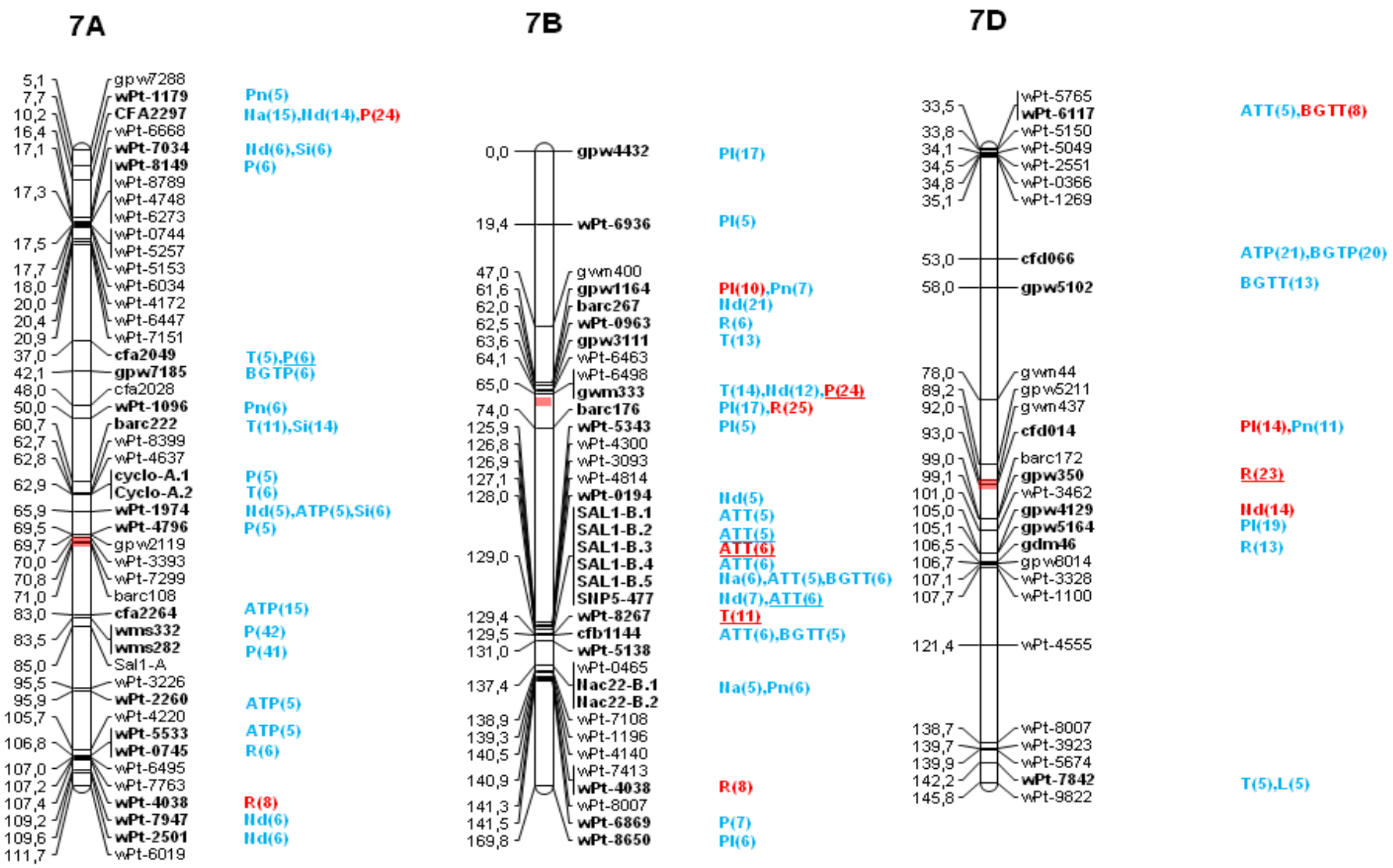
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Cyclo-A.2
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69,5 wPt-4796
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wms282
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ATP(5)
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SAL1-B.2
SAL1-B.3
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SAL1-B.5
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Na(5),Pn(6)
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P(7)
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93,0 cfd014
99,0 barc172
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BGTT(13)
Pl(14),Pn(11)
R(23)
Nd(14)
Pl(19)
R(13)
T(5),L(5)

2.3 Candidate gene association for vitamins composition

Candidate gene association mapping, which relates within candidate gene polymorphisms with phenotypic variations of the traits, is important to map targeted genes with known function (Tabor et al., 2002). In this study seventy-eight candidate genes (Figure 1),were associated with at least one vitamin. In addition 8 loci that did not include a candidate gene were in strong linkage disequilibrium with a candidate gene (To compare the region identified in the two populations, markers in our association map separated by less than 10 cM were considered in linkage desiquilibrium and part of the same QTL). The major wheat grain storage proteins (GSPs) are the glutenin and gliadin prolamins which make up 60-80% of total grain proteins. Glutenins are composed of high-molecular weight (HMW-GS) and low-molecular weight (LMW-GS) subunits. Wheat prolamins are encoded by several loci on the homologous groups one and six chromosomes (Shewry and Halford, 2003). Plessis et al. (2013) developed SNP markers in five HMW-GS and one LMW-GS genes. In this study, these SNP markers which have been used to genotype all the accessions of the core collection were significantly associated with vitamins. The HMW-GS genes GluB1.1, GluB1.2 and GluB1.3 were associated with pantothenic acid content. In addition on chromosome 1BL, one marker (wPt2315) mapping near GluB1.3 was also associated with pantothenic acid contents. GluD1.1, GluD1.2, GluD1.3 and GluD1.4 were associated with nicotinamide and β-γ-tocopherol contents. A marker (wPt3743) mapped near GluD1.1 was also associated with nicotinamide contents. Among the thirteen wheat orthologues of the barley transcriptor factors controlling GSPs gene expression for which polymorphic SNPs were found (Supplementary data 3) , only one (storage protein activator (SPA) of these candidate genes was associated with different vitamin traits. SPA-1A, SPA-1B and SPA-1D were associated ($p < 0.01$) with β-γ-tocotrienol, nicotinamide, lutein and thiamin contents. 16 putative genes of the transcription factors family members were also studied for genetic association. Nac22-B and Nac27-A on chromosomes 7BL and 5AL, respectively were associated with nicotinic acid and pyridoxine and with lutein content, respectively. DOF9-A on 5AL and 6AL was associated with α-tocopherol, β -γ-tocopherol, β-γ-tocotrienol, α-tocotrienol in Clermont-Ferrand and with lutein in both location. In Clermont-Ferrand, DOF19-B on 1BL and on 5BL was specifically associated with lutein, β-γ-tocopherol, pyridoxal and pyridoxine respectively. Platz4-A was significantly associated (r^2=9% and 11%, $p = 4.5x10^{-4}$) with thiamin and β-γ-tocopherol contents, respectively. Interestingly, for Platz4-A the average thiamin contents were highest when the individuals were carrying the allele G. Whereas, the average β-γ-tocopherol contents were highest when the individuals were carrying the allele A (Supplementary data 4). Furthermore, Platz4-A on 3AL was in linkage disequilibrium with a marker (Gpw7213) associated with pantothenic acid content. Because plants are constantly exposed to a variety of microbial pathogens, they have develop a battery of transcription factors. One of them is the DNA binding proteins containing WRKY60 domains (Ulker and Somssich, 2004). WRKY60 on 2BL was associated with β-γ-tocotrienol. The putative abscissic stress ripening protein (ASR4-B) gene on chromosome 2BS and the powdery mildew (PM) gene on chromosome 1BS was strongly associated (r^2 of 10%) with beta-sitosterol. Moreover, for PM1.2 the average beta-sitosterol contents were highest when the individuals were carrying the allele C. In Clermont-Ferrand the basic helix-loop-helix (bHLH) putative gene on 3AL was associated with lutein content. Finally,

among the wheat genes which enhance folate contents, wheat 6-hydroxymethyl-7,8 dihydropterin pyrophosphokinase (HPPK-B) on the long arm of chromosome 2B was significantly associated in Clermont-Ferrand with lutein (r^2 of10%, p = 1. 5x10^{-4}) and was in strong linkage disequilibrium with a marker (wPt7408) associated with nicotinamide. Furthermore, for HPPK-B the average lutein contents were highest when the individuals were carrying the allele A (Supplementary data 4). Of the six key genes controlling grain development, only SAL1-B, Vp1-B and Lec-1 showed significant association with vitamin traits. In both growing locations, SAL1-B showed consistent association with the concentration of α-tocotrienol. For SAL1-B, in le Moulon, the individuals carrying the allele C had a significantly higher content of α-tocotrienol, whereas in Clermont-Ferrand, the allele T of SAL1-B lead to an increased in the average contents of α-tocotrienol (Table3, Supplementary data 4). Moreover, SAL1-B was in strong linkage disequilibrium with a marker (wPt8267) strongly and significantly associated with thiamin content (r^2 of 11%, p = 3.10x10^{-5}).At Clermont-ferrand, SAL1-A on chromosome 7AL was in strong linkage disequilibrium with markers (Wms332 and Wms 282) strongly associated with pantothenic acid (r^2 of 42% and 41%). As demonstrated in 2.2.5, for Wms282, the individuals carrying the allele 220 bp and for Wms332 the individuals carrying the allele 234 bp had a significantly higher content of pantothenic acid. Vip-3A on chromosome 3AL was also significantly associated with α-tocotrienol (p = 7.1x10^{-3}), whereas Vip1-B on chromosome 3B was associated with α-tocopherol and vitamin B3. Vitamin B6 was strongly associated (r^2 of 10%) with Lec1-A on chromosome 3AS. Lec1-A was also in linkage disequilibrium with a marker (wPt6012) significantly associated with pantothenic acid.

PSY1 and PSY2 candidate genes

Psy2, the second gene encoding phytoene synthase showed to be associated with lutein on 5AS and with pyridoxine and α-tocotrienol on 5BS. However, a marker (gwm415) not far from Psy2-A (6 cM) was strongly associated with lutein content (r^2 of 13%; p = 8.1x10^{-4}). It was very surprising not to detect any associations between PSY1-A and PSY1-B on chromosomes 7. Ravel et al. (2013), have shown the influence of PSY1-A, PSY1-B and PSY1-D on the yellow pigment content (YPC) in the worldwide core collection. However, in wheat grains YPC reflect the total content of carotenoids plus additional compounds not yet identified. In this study, only free lutein content was considered for association mapping. In fact, in durum wheat among xantophylls, free lutein accounts for about 84.8 % of the yellow pigments, lutein monoesters account for 9.8 %, and lutein diesters account for 5.3 % (Lepage et al., 1968).Thus, it is possible that PSY2 genes strongly influence the accumulation of free lutein, whereas PSY1-genes might favor the accumulation of lutein as well as the other carotenoids. Apart from carotenoids pigments which have been widely studied in durum and bread wheats, genetic analyses of vitamin contents based on molecular markers in common wheat are very scarce. In this study, significant marker-trait associations for vitamin contents were detected on all chromosomes of the bread wheat through association mapping approach, indicating the complexity of vitamin content inheritance.

GluB and GluD candidate genes

Interestingly, regions significantly associated with pantothenic acid and nicotinamide traits were mapped in the region of known glutenin loci. Because pantothenic acid and nicotinamide are both compounds containing nitrogen, it might be possible that the synthesis of glutenin through the action of GluD1 and/or GluB1 genes might limit the availability of nitrogen for the synthesis of these two vitamins. Thus, at Le Moulon, the average pantothenic acid contents were highest when the individuals were carrying the allele G (Supplementary data 4). It would be therefore useful to study if a nitrogen competition occurs during grain development between storage proteins and those two vitamins. This possible competition is reinforced by the fact that both pantothenic acid and nicotinamide are strongly influenced by location effect (Chapter 6 paragraph 2.1.3).

Transcription factors (TFs) candidate genes

Abiotic stresses such as drought, salinity and low temperature adversely affect the growth and productivity of the plants. Identification and functional study of stress responsive genes may help to elucidate the molecular mechanisms of the plant stress response. The expression of functional proteins, such as enzymes for the removal of reactive oxygen species is widely regulated by specific TF (Sing and al., 2002; Rahaie and al., 2013).In our study, most of the association between vitamins and TFs such as Nac22, Nac27, ASR4, Dof9, Dof19 and bHLH occurred at one location (Clermont-Ferrand) where wheat cultivars were exposed to drought stress (Chapter 3,Table 3). Furthermore, the vitamins (tocopherols, tocotrienols, lutein, pyridoxine and sistosterol) involved in these associations are well known to possess strong antioxidant and radical scavenging activity. Thus, it could be hypothesized that drought stress-responsive TFs might be involved in the regulation of the expression these vitamins in bread wheat and that these TFs are potentially important regulators of large number of target “stress-responsive” genes for wheat adaptation to drought stresses.

Supernumerary aleurone layer (SAL) candidate genes

The aleurone layer of cereal grains is important biologically as well as nutritionally. Indeed, the aleurone layer is rich in proteins and contains the major part of the B vitamins and about half the total mineral content (Antoine et al., 2002; Pomeranz, 1988). Aleurone layer is a living cell layer in mature grain, where numerous enzymes and globulins (storage proteins) are expressed together with minerals and vitamins. Proteomic analyses revealed that the diversity of proteins, expressed in aleurone layer, was not strongly influenced by environmental conditions (Meziani et al., 2012, 2014). This cell layer is the site where numerous enzymes involved in stress/defense are increasingly expressed during grain formation (Nadaud et al., 2015). It has been shown (Shen et al., 2003) that mutants of the SAL1 gene have multiple layers of aleurone cells in maize instead of the normal single layer, indicating that the gene, functions as a negative regulator of aleurone fate. In our study strong and consistent associations were found between putative gene SAL-B and α-tocotrienol content. In addition strong linkage disequilibrium was observed between a marker (wPt8267) significantly associated with thiamin content and SAL-B. As

explained in 2.2.1, for DArT wPt 8267, in both locations the individuals carrying the allele wPt 8267 had a significantly ($p<0.05$) higher thiamin content than those not carrying this allele. The presence of SAL-B gene might contribute to the change of the thiamin and α-tocotrienol levels in the unique aleurone layer of the wheat grain.Furthermore, it was also noticed that under drought stress environment (Chapter 3,Table 3), phenotypic variation of pantothenic trait was strongly related to the presence of the SAL-A putative gene in the long arm of chromosome 7A.

2.4 Colocation of significant associations of several vitamins

Determination of very complex traits like vitamin contents is likely to involve many acting and interacting genes. On chromosome 3BS, the SSR marker (Gpw7452) was associated with thiamin, pyridoxine and pantothenic acid content at Clermont-Ferrand. In addition, at the same location, on chromosome 5BL, the DArT marker (wPt1733) which was associated with thiamin content was in strong linkage disequilibrium with the putative gene DOF19-B associated with pyridoxal and pyridoxine. These associations are consistent with the correlation observed at the same location (Chapter 6-Table 8) between thiamin and pyridoxine ($r =0.3527$, $n=195$) and between pyridoxine and pantothenic acid ($r = 0.2491$, $n=195$). Furthermore on chromosome 3BS, the DArT marker wPt 9170 was correlated with pyridoxine and pantothenic acid. The contents of nicotinic acid and nicotinamide were both weakly correlated with thiamin at Clermont-Ferrand (Chapter 6-Table 8). Interestingly, on chromosome 7BS, thiamin and nicotinamide content were strongly and significantly associated with Gwm333. In this study, the association of some markers, with several lipid-soluble compounds (on chromosome 1B, wPt5067, wPt4434 and wPt7529 was associated with β-γ-tocotrienol and lutein; on chromosome 5AS, Gwm415 was strongly assoiciated with α-Tocotrienol, β-Sitosterol and lutein contents; on chromosome 6AL, DOF9-A was associated with α-Tocotrienol, β-γ-tocotrienol and lutein content) might explained the correlation observed between these compounds (Chapter 6-Table9). Beside the stable associations (consistent over the two locations), the above associations may also result from environmental influences which were all highly significant for each vitamins (location effect shown on Chapter 6, Table 2). Future analyses could be developed in taking into account covariates of important traits like protein content or TKW as being partly associated to the grain morphology and its surface envelopes / volume ratio. In addition these future investigations could advantageously develop the present AM approach for the vitamin level expressed by grain.

3. Conclusion

Our results suggest that association mapping can be used to identify relevant genomics regions for vitamin traits using a worldwide bread wheat core collection. As suggested by Bordes et al. (2011), the core collection can also be used to test candidate genes using SNPs. In this study, several regions involved in the genetic control of complex vitamin traits were identified. Several TFs of unknown function in grain were involved in this genetic control and detected under heat

stress condition. Thus the associations between vitamins and the different TFs need to be deeply investigated and if confirmed these TFs, could be used as markers of drought stress. Furthermore, the strong association between SAL gene candidate and α-Tocotrienol and thiamin content may reflect a strong accumulation of these vitamins in the aleurone layer of wheat grain. Functional studies of these TFs and SALs genes are needed in the future. The current analysis provides innovative and relevant genetic data that can be used for future investigation on genetics of vitamin in wheat grain and devising the plant breeding strategies and oriented to improving vitamin content through marker assisted selection.

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Supplementary data

Supplementary data 3. Gene description, symbol and chromosome location of the putative candidate genes for which genetic association with vitamin contents have been analyzed. The name of the single nucleotide polymorphism markers reported in the association map (Figure 1) is the gene symbol. (from Bordes et al.,2011; Plessis et al.,2013).

Gene symbol	Gene full name	Chromosome
PBF-A	Prolamin-Box binding Factor	5AL
PBF-B		5BL
DHPA	Dihydroxypropyladenine	7BS, 1AL
SPA-1A	Storage protein activator	1AL
SPA-1B		1BL
SPA-1D		1DL
MCB1-A	Multiubiquitin Chain Binding Protein	1AL
MCB1-B		1BL
MYBS3-A	MYBS3 transcription factor	1AL
MYBS3-B		1BL
GAMYB-B	Gibberellic acid-dependant-MYB transcription factor	3BL
GAMYB-D		3DL
SAD-A	Scutellum and Aleurone expressed DOF transcription factor	6AL
SAD-B		6BL
WRKY60-B	WRKY transcription factor	2BL
PLATZ4-A	Plant AT-rich sequence – and Zinc- binding protein 4	3AL
HSF4-B	Heat-shock transcription factor 4	5BL
bHLH	Basic helix-loop-helix	3AL
ASR4-B	Abscissic Stress Ripening protein 4	2BS
DOF9-A	DNA binding with One finger protein 9	6AL
DOF19-A	DNA binding with One finger protein 9	1BL
DOF19-B		5BS
bZIP21-B	Basic leucine-ZIP domain-containing protein 21	2BL
NAC22-A	NAC domain-containing-	7AL

	protein 22	
Gene symbol	Gene full name	Chromosome
NAC22-B	NAC domain-containing-protein 22	7BL
NAC27-A	NAC domain-containing-protein 22	5AL
NAC27-B		5BL
MADS19-B	MCM1, AGAMOUS, DEFICIENS, and SRF domain-containing protein 19	2BL
MADS33-B	MCM1, AGAMOUS, DEFICIENS, and SRF domain-containing protein 33	1BL
DREB1-B	Dehydratation-Responsive Element Binding protein B1	3BS
HAP2-B	HAPless 2 protein	4BL
Dr1-D	Down-Regulator of transcription 1	3DL
PcG3-A	Histone-Lysine N-methyltransferase	4AL
PcG3-B		4BL
PHD20-B	Plant Homeo Domain-containing protein 20	4BL
SAL1-A	Supernumerary ALeurone Layer 1	7AL
SAL1-B		7BL
SAL1-D		7DL
Vip1-B	B3 domain-containing transcription factor viviparous 1	3BL
Vip-3A		3AL
FUS3-B	B3 domain-containing transcription factor FUS3	3BL
LEC1-A	Leafy Cotyledon 1 transcription factor	3AS
AAP-A	Amino Acid Permease	2AL
AAP-B		2BS
AAP-D		2DL
Dek1-A	Cysteine protease	6AL
Dek1-B		6BL
GAD1-B	Glutamate Decarboxylase	3BS
GDH1-A	Glutamate dehydrogenase	5AL
GDH1-B		5BL

GDH1-D		3DS
FdGOGAT-B	Ferredoxin-dependant glutamate synthase	2BS

Gene symbol	Gene full name	Chromosome
FdGOGAT-D	Ferredoxin-dependant glutamate synthase	2DS
NAM-B1-B	No Apical Meristem domain-containing protein B1	6BL
GluA1.1	High molecular weight glutenin	1AL
GluB1.1	High molecular weight glutenin	1BL
GluD1.1	High molecular weight glutenin	1DL
AAP-2A	Amino acid permease	2A
AAP-2B		2B
ZDS	Lycopene synthase	2A
LDD	Lumini dependens	3A
Gigantea	Gigantea	3A
BPGIPGM	Bishosphoglycerate independant Phosphoglycerate mutase	3B
LDD	Lumini dependens	3B
SPA-1A	Storage protein activator	1AL
SPA-2A		5A
SPA-1B		1BL
SPA-2B		5B
SPA-1D		1DL
Psy2-A	Phytoene synthase 2	5AS
Psy2-B		5BL
Psy2-D		5D
Hip	Hedgehog interacting protein	5AS
CHS	Chalcone synthase	5A
GSP-A	Grain softness protein	5AS
GSP-B		5BS
Pinb	Puroindoline B	5DL
Vern-B	Vernalization	5BS
PM	Powdery mildew	3AL,1BS
HPPK-B	6-hydroxymethyl-7,8 dihydropterin pyrophosphokinase	2BL

Supplementary data 4. Analysis of trait performance relevant to different alleles of significant loci reported on table 2 and 3.

Vitamin (Le Moulon)	Marker and polymorphism	Allele(bp)	Mean (µg/g DM)±SEM	Vitamin (Clermont-Ferrand)	Marker and polymorphism	Allele(bp)	Mean (µg/g DM) ±SEM
Thiamin	wPt8267	1	5,61±0.13^{A}	**Thiamin**	wPt8267	1	4.88±0.13^{A}
		0	4,97±0.05^{B}			0	4.35±0.05^{B}
	Platz4_A_R36 (G/A)	G	5.11±0.05^{A}				
		A	4.84±0.11^{B}				
					Gpw7452	127	4.53±0.16^{a}
						129	4.32±0.14^{a}
						137	4.77±0.23^{a}
						139	4.38±0.07^{a}
						141	4.49±0.08^{a}
					Barc222	194	5.06±0.31^{a}
						200	4.33±0.11^{b}
						202	4.88±0.17^{a}
						204	4.41±0.18^{b}
						206	4.21±0.11^{b}
					Gpw3111	187	4.43±0.10^{b}
						191	5.32±0.29^{a}
						193	4.13±0.16^{b}
						195	4.37±0.05^{b}
						197	4.30±0.17^{b}
					Gwm333	165	5.22±0.44^{a}
						167	4.33±0.06^{b}
						169	4.53±0.09^{b}
						171	4.37±0.12^{b}

Vitamin (Le Moulon)	Marker and polymorphism	Allele(bp)	Mean (µg/g DM)±SEM	Vitamin (Clermont-Ferrand)	Marker and polymorphism	Allele(bp)	Mean (µg/g DM) ±SEM
Pyridoxal	wPt4290	1	0.69±0.02^{B}	**Pyridoxal**			
		0	0.79±0.09^{A}				
					Gpw1164	207	0.82±0.01^{a}
						209	0.94±0.04^{a}
						213	0.85±0.06^{a}
	Lec1-A	T	0.63±0.02^{A}				
		C	0.66±0.04^{A}				
	Cfd8	173	0.60±0.08^{a}				
		175	0.65±0.02^{a}				
		177	0.61±0.02^{a}				
		179	0.63±0.04^{a}				
		183	0.60±0.07^{a}				
	Gwm219	150	0.56±0.07^{a}				
		165	0.75±0.15^{a}				
		167	0.77±0.04^{a}				
		173	0.56±0.05^{a}				
		175	0.61±0.10^{a}				
		179	0.67±0.04^{a}				
		183	0.57±0.07^{a}				

Vitamin (Le Moulon)	Marker and polymorphism	Allele(bp)	Mean (µg/g DM)±SEM	Vitamin (Clermont-Ferrand)	Marker and polymorphism	Allele(bp)	Mean (µg/g DM) ±SEM
Pyridoxal	Gwm219	185	0.63±0.05[a]	**Pyridoxal**			
		187	0.71±0.11[a]				
		189	0.70±0.05[a]				
		191	0.64±0.02[a]				
Pyridoxal	Gpw4432	241	0.64±0.04[a]	**Pyridoxal**			
		242	0.63±0.12[a]				
		243	0.58±0.04[a]				
		246	0.74±0.13[a]				
		249	0.63±0.02[a]				
		251	0.52±0.05[a]				
Pyridoxal	Cfd014	138	0.69±0.04[a]	**Pyridoxal**	L		
		140	0.64±0.11[a]				
		142	0.63±0.02[a]				
		144	0.61±0.02[a]				
		146	0.79±0.24[a]				
Pyridoxine	Gwm135	50	1.11±0.08[a]	**Pyridoxine**			
		139	1.07±0.03[a]				
		141	1.15±0.03[a]				
		151	1.05±0.1[a]				
		182	1.19±0.16[a]				
		196	1.30±0.09[a]				

Vitamin (Le Moulon)	Marker and polymorphism	Allele(bp)	Mean (µg/g DM)±SEM	Vitamin (Clermont-Ferrand)	Marker and polymorphism	Allele(bp)	Mean (µg/g DM) ±SEM
Pyridoxine	Lec1-A	T	1.10±0.02^{A}	**Pyridoxine**			
		C	1.09±0.06^{A}				
	Gwm261	165	1.08±0.05^{a}				
		175	1.08±0.03^{a}				
		193	1.17±0.07^{a}				
		197	0.97±0.09^{a}				
Pyridoxine				**Pyridoxine**	Barc147	121	1.09±0.04^{bc}
						123	1.05±0.02^{bc}
						125	1.28±0.07^{a}
						141	0.92±0.05^{c}
					Gpw7452	127	1.09±0.07^{b}
						129	1.11±0.08^{b}
						137	1.37±0.12^{a}
						139	1.06±0.02^{b}
						141	1.05±0.02^{b}
	wPt6688	1	1.07±0.03^{A}				
		0	1.05±0.03^{A}				
	wPt5505	1	1.13±0.04^{A}				
		0	1.06±0.03^{A}				

Vitamin (Le Moulon)	Marker and polymorphism	Allele(bp)	Mean (µg/g DM)±SEM	Vitamin (Clermont-Ferrand)	Marker and polymorphism	Allele(bp)	Mean (µg/g DM) ±SEM
Pyridoxine	Cfd014	138	1.13±0.06^{a}	**Pyridoxine**			
		140	1.11±0.14^{a}				
		142	1.11±0.05^{a}				
		144	1.06±0.03^{b}				
		146	1.37±0.24^{a}				
Nicotinamide	PGK-1D.R.1804(G/A)	G	1.18±0.03^{B}	**Nicotinamide**			
		A	1.34±0.04^{A}				
					wPt6973	1	0.49±0.01^{A}
						0	0.46±0.01^{B}
					wPt0373	1	0.46±0.01^{B}
						0	0.52±0.01^{A}
Nicotinamide				**Nicotinamide**	Barc267	161	0.47±0.03^{b}
						165	0.49±0.06^{b}
						167	0.49±0.02^{b}
						169	0.43±0.03^{b}
						171	0.61±0.06^{a}
						173	0.44±0.01^{b}
						175	0.44±0.02^{b}
						177	0.46±0.02^{b}
						179	0.46±0.02^{b}
						181	0.52±0.02^{ab}
						183	0.52±0.03^{ab}

Vitamin (Le Moulon)	Marker and polymorphism	Allele(bp)	Mean (µg/g DM)±SEM	Vitamin (Clermont-Ferrand)	Marker and polymorphism	Allele(bp)	Mean (µg/g DM) ±SEM
Nicotinamide				**Nicotinamide**	Barc267	184	0.49±0.02[b]
						186	0.49±0.04[b]
						188	0.51±0.06[ab]
						192	0.48±0.03[b]
	Gpw7384	225	1.27±0.02[B]				
		227	1.49±0.13[A]				
	Gwm333	165	1.20±0.08[a]				
		167	1.26±0.03[a]				
		169	1.33±0.06[a]				
		171	1.25±0.07[a]				
					Gpw4129	339	0.49±0.01[A]
						241	0.46±0.01[A]
Riboflavin				**Riboflavin**	wPt1911	0	0.78±0.03[A]
						1	0.80±0.02[A]
					Gwm261	165	0.83±0.03[a]
						175	0.81±0.02[a]
						193	0.81±0.04[a]
						197	0.69±0.06[a]
	wPt9154	0	0.85±0.04[B]				
		1	1.23±0.16[A]				
	wPt5914	0	0.73±0.07[A]				
		1	0.89±0.04[A]				
					wPt2707	0	0.79±0.02[A]
						1	0.82±0.02[A]

Vitamin (Le Moulon)	Marker and polymorphism	Allele(bp)	Mean (µg/g DM)±SEM	Vitamin (Clermont-Ferrand)	Marker and polymorphism	Allele(bp)	Mean (µg/g DM) ±SEM
Riboflavin				**Riboflavin**	wPt8006	0	0.87±.0.02^{A}
						1	0.76±0.02^{B}
					wPt9976	0	0.89±0.04^{A}
						1	0.77±0.02^{B}
					wPt5333	0	0.78±0.01^{B}
						1	0.87±0.04^{A}
	Gpw7433	310	0.74±0.03^{B}				
		337	1.35±0.33^{A}				
	wPt4038	1	0.89±0.04^{A}				
		0	0.76±0.04^{A}				
Riboflavin	Gpw350	191	0.77±0.06^{b}	**Riboflavin**	Gpw350	191	0.78±0.05^{b}
		216	0.86±0.23^{ab}			216	0.81±0.06^{b}
		218	1.24±0.19^{a}			218	1.11±0.09^{a}
		220	0.76±0.06^{b}			220	0.83±0.03^{ab}
		222	1.06±0.11^{ab}			222	0.73±.0.04^{b}
		224	0.76±0.17^{b}			224	0.79±0.06^{b}
		225	0.84±0.05^{b}			225	0.79±0.02^{b}
		228	0.75±0.08^{b}			228	0.75±0.05^{b}
		229	0.91±0.11^{ab}			229	0.75±0.07^{b}
		231	1.04±0.34^{ab}			231	0.91±0.13^{ab}

Pantothenic acid	wPt6012	0	3.91±0.08^{A}	**Pantothenic acid**			
		1	3.57±0.15^{A}				
	GluB1.R.427 (A/G)	G	4.16±0.18^{A}		GluB1.R.427 (A/G)	G	8.17±0.22^{A}
		A	3.75±0.08^{B}			A	7.99±0.15^{A}
Vitamin (Le Moulon)	Marker and polymorphism	Allele(bp)	Mean (µg/g DM)±SEM	Vitamin (Clermont-Ferrand)	Marker and polymorphism	Allele(bp)	Mean (µg/g DM) ±SEM
Pantothenic acid				**Pantothenic acid**	Barc147	121	8.32±0.30^{a}
						123	7.76±0.14^{a}
						125	8.12±0.41^{a}
						141	8.31±0.48^{a}
	Gpw7452	127	3.87±0.29^{b}		Gpw7452	127	8.22±0.54^{b}
		129	3.52±0.29^{b}			129	7.29±0.12^{b}
		137	5.14±0.83^{a}			137	10.08±1.73^{a}
		139	3.68±0.09^{b}			139	7.69±0.17^{b}
		141	3.86±0.1^{b}			141	8.12±0.16^{b}
Pantothenic acid	Barc164	195	4.25±0.36^{ab}	**Pantothenic acid**	Barc164	195	7.95±0.31^{b}
		198	3.62±0.1^{b}			198	7.75±0.18^{b}
		200	3.76±0.12^{b}			200	7.97±0.23^{b}
		206	4.59±0.32^{a}			206	9.26±0.54^{a}
		210	3.82±0.15^{b}			210	8.08±0.24^{ab}
					Gwm190	202	7.55±0.26^{a}
						206	7.29±0.41^{a}
						208	7.47±0.38^{a}
						210	8.10±0.29^{a}

						212	8.11±0.20[a]
						214	7.81±0.26[a]
						216	7.97±0.49[a]
					Wms332b	218	8.71±0.61[ab]
						224	8.23±0.65[b]
						226	7.52±0.26[b]
Vitamin (Le Moulon)	Marker and polymorphism	Allele(bp)	Mean (µg/g DM)±SEM	Vitamin (Clermont-Ferrand)	Marker and polymorphism	Allele(bp)	Mean (µg/g DM) ±SEM
Pantothenic acid				**Pantothenic acid**	Wms332b	228	7.66±0.39[b]
						230	8.35±0.49[b]
						232	7.65±0.28[b]
						234	10.14±0.83[a]
						238	7.76±0.19[b]
						253	7.37±0.44[b]
						263	7.34±0.31[b]
						265	7.84±0.07[b]
						298	7.91±0.45[b]
Pantothenic acid				**Pantothenic acid**			
					Wms282	204	8.71±0.61[ab]
						210	8.23±0.65[b]
						212	7.52±0.26[b]
						214	7.72±0.36[b]
						216	8.35±0.49[b]
						218	7.65±0.28[b]
						220	10.14±0.83[a]
						224	7.76±0.19[b]
						239	7.37±0.44[b]
						249	7.34±0.31[b]
						251	7.84±0.07[b]

						284	7.91±0.45[b]
	Gwm333	165	3.50±0.33[a]		Gwm333	165	8.03±0.98[ab]
		167	3.73±0.09[a]			167	7.71±0.12[b]
		169	3.97±0.15[a]			169	8.36±0.26[a]
		171	3.93±0.18[a]			171	8.23±0.38[ab]

Vitamin (Le Moulon)	Marker and polymorphism	Allele(bp)	Mean (µg/g DM)±SEM	Vitamin (Clermont-Ferrand)	Marker and polymorphism	Allele(bp)	Mean (µg/g DM) ±SEM
α-tocopherol	CFA2264	322	3.67±0.26[abc]	**α-tocopherol**			
		324	3.22±0.12[c]				
		326	2.96±0.53[c]				
		328	3.37±0.07[bc]				
		330	3.64±0.23[abc]				
		334	3.92±0.15[ab]				
α-tocopherol	cfd066	174	3.67±0.14[ab]	**α-tocopherol**			
		176	3.51±0.23[abc]				
		178	4.02±0.34[ab]				
		180	2.92±0.21[c]				
		184	3.38±0.33[abc]				
		186	3.42±0.27[abc]				
		188	3.51±0.11[abc]				
		190	4.03±0.30[a]				
		192	3.19±0.23[bc]				
		194	3.72±0.18[ab]				
		196	3.59±0.30[abc]				
		198	3.03±0.20[bc]				
		202	3.28±0.33[abc]				

β-γ-tocopherol	Platz4_A_R100(A/G)	A	13.19±0.37^{A}	**β-γ-tocopherol**			
		G	11.02±0.29^{B}				
	39310-3B.M.212(A/C)	A	11.15±0.25^{B}				
		C	13.66±0.42^{A}				
	Gwm427	198	11.69±1.19^{a}				
		200	11.98±0.51^{a}				
		212	12.07±0.46^{a}				
		214	12.18±0.47^{a}				
Vitamin (Le Moulon)	Marker and polymorphism	Allele(bp)	Mean (µg/g DM)±SEM	Vitamin (Clermont-Ferrand)	Marker and polymorphism	Allele(bp)	Mean (µg/g DM) ±SEM
β-γ-tocopherol	Gwm427	216	11.57±0.62^{a}	**β-γ-tocopherol**			
		218	11.54±0.89^{a}				
		220	12.48±1.08^{a}				
β-γ-tocopherol	Cfd066	174	13.25±0.14^{a}	**β-γ-tocopherol**			
		176	10.97±0.23^{abc}				
		178	13.13±0.34^{a}				
		180	9.81±0.21^{c}				
		184	12.10±0.33^{abc}				
		186	10.62±0.27^{bc}				
		188	12.78±0.11^{a}				
		190	11.99±0.30^{abc}				
		192	11.77±0.23^{abc}				
		194	12.21±0.18^{abc}				
		196	11.65±0.30^{abc}				
		198	11.39±0.20^{abc}				
		202	13.14±0.32^{a}				

α-tocotrienol	Vip-3A.Y.85(C/T)	C	1.44±0.03[A]	**α-tocotrienol**			
		T	1.36±0.04[A]				
	Gwm415	129	1.30±0.18[a]		Gwm415	129	0.74±0.13[a]
		131	1.39±0.06[a]			131	0.68±0.05[a]
		133	1.41±0.03[a]			133	0.65±0.02[a]
		135	1.43±0.09[a]			135	0.71±0.05[a]
					Gwm272	135	0.75±0.11[a]
						137	0.64±0.03[a]
						139	0.69±0.07[a]
						145	0.65±0.04[a]

Vitamin (Le Moulon)	Marker and polymorphism	Allele(bp)	Mean (µg/g DM)±SEM	Vitamin (Clermont-Ferrand)	Marker and polymorphism	Allele(bp)	Mean (µg/g DM) ±SEM
α-tocotrienol	SAL-B-S9(C/T)	C	1.247±0.04[B]	**α-tocotrienol**	SAL-B-S9(C/T)	C	0.71±0.02[A]
		T	1.505±0.03[A]			T	0.60±0.03[B]
β-γ-tocotrienol	wPt8460	0	11.64±0.28[A]	**β-γ-tocotrienol**	wPt8460	0	10.72±0.28[A]
		1	9.89±0.30[B]			1	8.69±0.3[B]
	Gpw5102	161	9.50±0.85[a]				
		170	11.40±0.26[a]				
		173	10.32±0.40[a]				
		179	12.18±1.65[a]				
	Gwm160	196	9.91±0.50[b]				
		198	9.69±0.51[b]				
		202	11.44±0.25[b]				
		207	13.74±1.19[a]				
	Gwm251	101	9.66±0.93[b]				

		107	10.79±0.72[ab]
		109	12.14±0.56[a]
		111	11.17±0.47[ab]
		113	11.38±0.59[ab]
		115	11.49±0.69[ab]
		117	10.75±0.41[ab]
	wPt6117	0	12.49±0.85[A]
		1	10.94±0.22[B]

Vitamin (Le Moulon)	Marker and polymorphism	Allele(bp)	Mean (µg/g DM)±SEM	Vitamin (Clermont-Ferrand)	Marker and polymorphism	Allele(bp)	Mean (µg/g DM) ±SEM
β-sitosterol				**β-sitosterol**	PM1.2(T/C)	T	49.67±1.70[B]
						C	94.49±35.16[A]
					ASR4-B(A/G)	A	49.59±1.79[A]
						G	57.75±13.78[A]
β-sitosterol	Barc77	166	92.41±7.29[a]	**β-sitosterol**			
		178	74.49±8.34[abc]				
		182	55.01±6.02[c]				
		186	61.86±8.74[bc]				
		194	77.95±7.98[abc]				
		230	73.02±3.86[bc]				
		234	70.68±6.21[bc]				
		256	78.00±5.16[ab]				
	Gwm415	129	66.61±8.09[b]				
		131	76.00±4.28[ab]				
		133	73.64±2.77[ab]				
		135	90.73±9.49[a]				

					Gwm642	189	49.86±2.41[a]
						191	37.74±5.26[a]
						203	50.66±2.82[a]
						205	42.37±8.31[a]
					Barc222	194	45.19±7.82[a]
						200	38.16±5.33[a]
						202	41.83±6.16[a]
						204	50.36±1.97[a]
						206	53.26±3.98[a]

Vitamin (Le Moulon)	Marker and polymorphism	Allele(bp)	Mean (µg/g DM)±SEM	Vitamin (Clermont-Ferrand)	Marker and polymorphism	Allele(bp)	Mean (µg/g DM) ±SEM
Lutein	Gwm257	193	0.42±0.06[a]	**Lutein**	Gwm257	193	0.38±0.07[a]
		195	0.43±0.04[a]			195	0.36±0.04[a]
		197	0.44±0.03[a]			197	0.37±0.03[a]
		201	0.48±0.04[a]			201	0.36±0.09[a]
					HPPK-B(G/A)	G	0.35±0.02[B]
						A	0.60±0.18[A]
	Gwm415	129	0.35±0.03[a]		Gwm415	129	0.26±0.08[a]
		131	0.48±0.05[a]			131	0.39±0.05[a]
		133	0.42±0.03[a]			133	0.34±0.03[a]
		135	0.55±0.06[a]			135	0.50±0.06[a]
	WPt8266	0	0.40±0.02[A]		WPt8266	0	0.34±0.03[A]
		1	0.47±0.04[A]			1	0.37±0.03[A]

Differences in the vitamin contents for the different alleles of a SSR marker were evaluated using a LSD multiple comparisons test. Different superscript lower letters indicate statistically differences at the probability level < 0.05.

Differences in the vitamin contents for the two different alleles of a Dart or SNP markers as well as SSR markers with only two alleles were evaluated using a Student's t-test. Different superscript capital letters indicate statistically differences at the probability level < 0.05.

Note. For the DArT markers, the presence or the absence of individual fragments is represented by 0 (for absence) or 1 (for presence).

Allele (bp) is PCR product amplified by SSR markers.

Conclusion and Perspectives

Wheat as one of the major world agricultural products, has so far received great attention regarding its technological quality attributes but little work focuses on its nutritional quality. However, it is becoming evident that many of the health benefits associated with the consumption of whole wheat grain product relate to the enhanced intake of micronutrients and phytochemicals (Fardet, 2010). As a consequence, a number of studies have been devoted to the development of analytical methods which could explore and monitor the nutrient composition of whole-grain wheat or end-uses products. Nevertheless, in the objective of a large scale varietal screening, none of the reported methods were suitable for the analysis of wheat flour and wheat food products. Despite the potential of liquid chromatography/tandem mass spectrometry (LC-MS/MS), only a few methods have been applied for the determination of multivitamin in food samples. For this reason, the first objective of this thesis was to develop and validate a rapid and high throughput LC-MS/MS method for the determination of water and lipide-soluble compounds in various wheat-based food materials.

1. Evaluation of the developed analytical method

1.1. General conclusion

Water-soluble vitamins are important class of compounds that require quantification from food sources to monitor nutritional value. In this thesis, we developed a simple and rapid method based on liquid chromatography tandem mass spectrometry (LC-MS/MS) for the simultaneous determination of seven water-soluble vitamins in various wheat-based food materials. This approach was totally innovative and overcome the main difficulties reported in literature for the simultaneous analysis of this class of compounds in complex matrices such as wheat and wheat-based food products. The simplicity of the method allowed us to successfully evaluate the content of seven B vitamins of a large international bread wheat core collection of 196 accessions grown in two sites. However, most of the vitamins exist as groups of chemically related compounds having similar biological activity capable of meeting a nutritional requirement (frequently called “vitamers”). For the case of water-soluble vitamins, niacin (nicotinic acid + nicotinamide) and vitamin B6 (pyridoxine, pyridoxal and pyridoxamine) constitute an interesting case, in which, the glycosylated forms of pyridoxine and nicotinic acid are prevalent in plant-derived foods (Gregory et al., 1991). In addition, it was shown by Wall and Carpenter (1988) that in the milky kernel of maize, nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) were the predominant form in which niacin occurs. Common extraction processes for these vitamers involve alkali or acid hydrolysis under heating condition (either in a boiling water bath or in an autoclave). Such extraction release free vitamins from its bound forms. Nevertheless, the susceptibility of the other vitamins to degradation by exposure to heat and alkaline pH constrained us to achieve a softer extraction method which did not allow the complete release of the vitamins present in the sample as NAD,

NADP, nicotinic acid glucoside and pyridoxine glucoside. In data reported by Sampson et al. (1996), there was a significant fraction (average of 68%) of vitamin B6 in wheat present as pyridoxine glucoside. An important additional finding is the changes in the distribution of niacin compounds in corn during its development. Immature sweet corn is an effective source of NAD and NADP, whereas niacin in mature field corn is largely present as glycosylated forms of niacin (Wall and Carpenter, 1988). Thus, it will be of interest to develop a simple, reliable and high throughput analytical method which allows the measurement of all biologically active B-vitamers. Considering the analysis of the lipide-soluble compounds in our study, the used of a LC-MS/MS system coupled with an extraction method previously described (Lyan et al., 2001) has permitted the quantification of luteins, tocols derivatives and β-sitosterol in the different food matrices. Nevertheless, as lipophilic compounds, tocols are associated with lipid components of the sample matrix. As suggested by Panfili et al. (2003), there is an important need to add a saponification step which transforms the tocols esters into their corresponding alcohols to quantify total tocols from cereals. In addition, sterols can be found in four forms in cereal grains, namely, free, ester of fatty acids, hydroxycinnamic acid and conjugated with sugars (mostly with glucose, called sterol glycosides and acylated sterol glycosides). In hexaploid wheats it was reported (Lafelice et al., 2009) that the free sterol fraction represented 51% of the total sterol, whereas the esterified sterols and bound sterols fractions were 41% and 8% respectively. Among xanthophylls, free lutein accounts for 85% of the yellow pigments, lutein monoesters account for 10%, and lutein diesters account for 5% (Lepage et al., 1968). It was proposed (Panfili et al., 2004; Irakli et al., 2011) that saponification, involving heating of the sample in a strong alkaline environment, favors the release of carotenoids from their bounding forms with a reduced loss of analytes from the food matrix. To the light of the different results expressed in the literature the addition of a saponification step during the extraction procedure may provide higher liposoluble compounds recoveries. However, depending on the nature of the carotenoid and the food type, saponification may result in destruction or structural transformation (Olivier et al., 1998). Moreover, in the objective of a large scale varietal screening, adding a saponification step, which is time-consuming, is not suitable for the analysis of wheat flour and wheat based products. Considering these observations, we decided for the purpose of this thesis to include only a liquid-liquid extraction step in the developed procedure for a fast and simultaneous quantification of the free-liposoluble. Nevertheless, it might be useful in a near future to develop an analytical method which allows the determination of the different lipide-soluble compounds composition (total, free and esterified) in a single chromatographic run, in order to have a correct evaluation of the healthy and nutritional values of bread wheat samples of interest.

1.2. Perspectives about improving techniques

- Development of a high throughput analytical method for the measurement of all biologically active water-soluble vitamers

To achieve such goal, the important issue of extraction process has to be examined closely. Indeed, if we considered getting the nutritional composition of the free vitamers and the glycosylated forms, a two step extraction should be performed. The first extraction will aim to quantify the total vitamers (free, conjugated or phosphorylated) content by use of a vigorous

treatment with heat and mineral acids. Such treatment will hydrolyze the phosphorylated and the glycosylated forms to liberate the free vitamers. The second extraction (it requires a milder treatment with organic acids without heat and an enzymatic treatment to denature protein, to hydrolyze the phosphorylated forms and to cleave the N-ribosyl bond) will aim to release from the food matrices the glycosylated forms and the total free vitamins (free vitamins+ vitamins released from their phosphate or N-ribosyl linkage). The LC-MS/MS method developed during this thesis could be applied for the simultaneous quantification of the water-soluble vitamin content of both extracts. The differences between B-vitamins content of extract 1 and extract 2 should give us the varying amounts of glycosylated forms of pyridoxine and binding forms of vitamin niacin (NAD, NADP, nicotinic acid glucoside). However, even if this multistep extraction will be efficient to evaluate the nutritional status of B-vitamers in wheat, it might not be suitable in an objective of a large-scale varietal screening. Therefore, it will be interesting to take full advantage of the high sensitivity and selectivity of the MS/MS detection. Thus, the challenge will be to optimize the MS/MS parameters of the different binding forms of B-vitamins and to find chromatographic conditions which allow the separation of the binding and free forms of vitamins in a short run time with a good performance in terms of peak shape and peak intensity.

- Development of a high throughput analytical method for the measurement of all biologically active lipide-soluble compounds

Because it might be complicated to find available standard of the different binding form of lipide-soluble compounds, it should be judicious to develop a LC-MS/MS method which include a two steps extraction procedure prior to the HPLC analysis. The first extraction step would provide the content of free lipide-soluble compounds in the food sample (a simple liquid-liquid extraction, similar to the extraction procedure used in this thesis, will be sufficient). The second extraction step would provide the content of total lipide-soluble compounds by a simple saponification (to break up the fats and release the compounds) and liquid/liquid extraction. The LC-MS/MS methods developed in this thesis could be applied to both extracts. The differences between lipide-soluble compounds content of extract 2 and extract 1 should give us the varying amounts of binding forms of lipide-soluble compounds. Furthermore, like wheat grains contain all of the vitamin E vitamers, including β and γ isomers of tocopherols and tocotrienols, it might be of interest to test new stationary phases capable of separating these isomers. In our study, the reversed phase column used was not sufficiently efficient in separating β and γ isomers. In the literature it appeared that the C_{30} column provides an excellent separation for vitamin E and carotenoid stereo-isomers (Burkhardt and Böhm, 2007; Sander et al., 2000). However, its extended running time up to 80 min without equilibration time limited its application in the objective of a fast screening (Sander et al., 2000). Further investigations have to be considered to allow the separation of lipide-soluble compounds using this new column in less than 30 min.

- Application of near infrared reflectance spectroscopy (NIRS)

The use of high performance liquid chromatography (HPLC) methods coupled with a mass spectrometer detector has shown to be very useful for vitamin determinations. Nevertheless,

when using LC-MS/MS, difficulties due to time of extraction, complexity of laboratory analyses or cost of analysis may be a major limitation for breeding program (particularly for early breeding generations which implies a large number of samples to be screened). Less complicated than HPLC, NIRS is an accurate, non-destructive compounds methodology and facilitating analysis of several traits simultaneously. The HarvestPlus project has already explored the application of NIRS for rapid and inexpensive semi-quantitative screening of maize sample for provitamin A, and for screening maize and wheat for Fe and Zn (Ortiz-Monasterio et al, 2007). Efforts to develop NIRS calibration curves for vitamins should be further investigated and one can imagine that development of the calibration models for quantifying the wheat flour vitamins could be achieved using primary reference data obtained from LC-MS/MS methods.

Nowadays the nutritional value of cereals and cereal-based foods such as breakfast cereals or bread as affected by ingredients, storage, processing techniques (milling technologies and bread making) are well reviewed and tend to show the ways by which the nutritional quality of wheat product can be improved to maintain the potential health benefits of whole-grain wheat. Because of the lack of efficient and rapid biochemical analytic methods, little investigations have been conducted on the composition in bioactive compounds of the different wheat milling fractions produced during the various processing operations. Furthermore, knowledge about the impact of breadmaking on specific bioactive compounds is plethoric, while no information is available about the effect of toasting on B and E vitamins, Luteins and β-sitosterol composition of toasted bread. Thus, the second objective of this thesis was to determine by means of LC-MS/MS and stable isotope dilution assay (Nurit et al., 2015), these compounds in different industrial milled fractions to evaluate the fraction with the highest health benefits and secondly to compare the variability of these component contents at each step of the breadmaking process of toasted bread produced with semi-coarse wheat flours (French flour, type 110) obtained from an industrial milling company and a bakery company.

2. Evaluation of food processing on vitamin contents and biovailability

2.1. General conclusion

The results of our study have demonstrated that:

- The enriched fraction obtained with an improved industrial milling process showed promising results in the objective of being used as a functional ingredient in order to enrich wheat-based products in thiamin and in lipide-soluble compounds.
- The toasting process can release bound bioactive compounds and lead to enhance the nutritional quality of toasting bread.
- The pyridoxine and niacin in mature cereal grains might be present largely as chemically bound forms that are nutritionally unavailable. As a consequences, analytical methods which measure the total nicotinic acid and nicotinamide and pyridoxine and pyridoxal

(i.e., free plus bound) content of the food sample may be providing a gross overestimate of the biologically available form of these vitamins.

2.2. Perspectives about grain processing

- **Dry fractionation processes : Histological fractionation**

Until recently, wheat bran was considered to be a milling by-product and was mostly used in animal feed. However, micronutrients, phytochemicals and fiber fractions which are concentrated in the bran and germ (Fardet, 2010) have high value for human nutrition. Thus, new milling/grinding processes have emerged in order to separate the interesting parts of the bran of the undesirable ones (Hemery et al., 2007). The first approach called 'histological fractionation' aims to dissociate the different constitutive layers of bran to get fractions rich in only one particular tissue of interest (generally the aleurone layer). The analyses made during this thesis of the industrial enriched fraction (which could be enrich with the aleurone layer)have shown that it contains an important amount of free lipide-soluble compounds and thiamin. Nevertheless, the concentration of free niacin and pyridoxal and pantothenic acid was more important in the other bran fraction. Antoine et al. (2002) have collected data concerning the biochemical composition of the aleurone and reported higher value in niacin and pyridoxine than our study. The variability in these vitamins was accounted for by the wheat varieties x environmental growing condition probably influencing the metabolites in the sample analyzed and also by methodology used. In fact, Antoine et al. (2002) analyzed the total content of vitamin B6 and niacin (i.e., free plus bound) in the aleurone tissue. Thus, we could hypothesize that an important part of niacin and pyridoxine might be mainly present in the aleurone layer as binding compounds and more precisely as glycosylated forms which are nutritionally unavailable (Gregory et al., 1991; Wall and Carpenter, 1988). Further investigations would be necessary to better understand the distribution of bound vitamins within the different fractions. Histological fractionation might be an interesting perspective to enrich wheat-based product but an important effort have to be done in term of chemical analysis to genuinely ensure that the bioactive compounds present in the interesting parts of the bran are totally bioavailable. Otherwise, the enriched wheat-based product will not have any nutritional benefit for the consumers. Furthermore, in a near future combination of adapted pretreatments and processes applied to new cultivars should allow a substantial reduction in the production costs. In parallel to the development of new fractions and products with enhanced nutritional quality, special attention should be given to the nutritional labeling. As suggested by Gregory (2012), the objective of nutritional labeling and food composition databases has to be reevaluated in order to reflect nutritional properties to the greatest possible extent. As an example, for vitamin B6 and niacin in cereals, a system for expressing total available vitamin B6 and niacin adjusting for the incomplete bioavailability of glycosylated forms should be implemented. As mentioned earlier (paragraph 1.2), simple and accessible methods that allow the measurement of individual vitamers should be developed. The possibility of developing NIRS calibration curves for vitamins analysis would greatly facilitate and accelerate the work of the agro-food industry and cereal sector. Moreover, NIR process analysis of grain and flour to measure moisture, ash, starch damage, protein content is already used by flour millers worldwide.

In addition to milling process, the pretreatments such as pre-germination and/or pre-fermentation of grains and cooking conditions may greatly increase or decrease the density of bioactive compounds (Fardet, 2014; Batifoulier et al., 2005; Leenhardt et al., 2006).

- **Cooking conditions**

The results obtained during this thesis on the impact of the different stage of breadmaking have provided evidence that in general all vitamins were susceptible to loss from heat. However, our results also demonstrated that the toasting process can release bound bioactive compounds and lead to enhance the nutritional quality of toasting bread. Wall and carpenter (1988) pointed out that interest in some traditional ways of food processing such as boiling mature corn grain in alkaline wood ashes solution may provide better nutrition. In fact hot alkali treatment made corn niacin nutritionally available (Kodicek et al., 1956). More recently, Nelson et al. (2013) stated that the application of the germination processes may increased the health benefits and acceptability of whole grains. Actually, germination unlocks many nutrients like niacin (Ongol et al., 2013) which are in bound forms and so increase nutrient bio-availability. Thus, along the several issues mentioned by Fardet (2014), which have to be addressed at the level of "second transformation", the effects of kneading and baking on bound vitamins should be further investigated. Such studies will be particularly helpful for breeders and for the baking industry. As an example, we could imagine that a variety of wheat with high level of bound vitamin forms might be very useful to produce toasting bread enriched in those vitamins if baking or kneading has no effect on bound vitamins. Furthermore, in such wheat varieties, germination could also provide an interesting alternative to liberate the active vitamins from their binding forms with the objective of improving the nutritional content of traditional bread. Screening varieties to seek those that have the most binding or free vitamin forms should be addressed.

- **The bioavailability complexity**

A common definition for the term bioavailability in the nutritional context is the proportion of a nutrient that is absorbed from the diet and used for normal body functions. However, different factors can influence the bioavailability of a nutrient. For example in the case of cereal products, numerous factors linked to the composition of the food matrix may limit the liberation of the nutrient. In addition, mineral and other nutrients exist in different chemical forms in the food and hence may influence their bioavailability. The variation among vitamers may lead to an incomplete bioavailability of the B-vitamins. Very low bioavailability was observed for the bound forms of niacin in grains unless grains were subjected to an alkaline treatment before transformation in tortillas (Wall and Carpenter, 1988). The glycosylated forms of pyridoxine which range from 5% to 75% of the total vitamin B6 in fruits, vegetables and grains constitute an important factor that affects the overall bioavailability of vitamin B6. Iron is also a good example of how the different chemical forms found in the food can influence their bioavailability. Dietary iron has two main forms: heme and non-heme iron. The richest source of heme iron in the diet include meat, fish and poultry, whereas dietary sources of nonheme iron include nuts, beans, vegetables and grain products. It is well known that heme iron has higher

bioavailability than nonheme iron, and that other components from the diet have more effect on the bioavailability of nonheme than heme iron (Hurrell and Egli, 2010). Because, heme is a cofactor consisting of an ferrous ion contained in the center of a large heterocyclic organic ring called porphyrin, it shields the iron from interaction with other food components and thus it is absorbed intact on the surface of the gut cell. On the other hand, the nonheme iron is very sensitive to chelators such as phytate or certain polyphenols with which they formed different types of insoluble complex that are unavailable for absorption and as a consequence decrease the bioavailability of nonheme iron. While, some inhibitors may reduce nutrient bioavailability, others like ascorbic acid may act as enhancer of nonheme iron absorption (Teucher et al., 2004).

Other factors such as gender, age, nutrient status and life stage (e.g. pregnancy) may either favor or impair the bioavailability of nutrients. It is evident from this list of factors that trying to give only a simple definition of the bioavailability of a nutrient is a very complex task. Therefore, some definitions of bioavailability restrict themselves to the fraction of a nutrient that is absorbed (Jackson, 1997). However, a comprehensive evaluation of this concept may be beyond the understanding of the public or of the food industries. It should be interesting as proposed by Gregory (2012) in the near future to develop generalized approaches to account for differences in bioavailability of vitamers' and to thoroughly enlighten the consumer about the nutritional labeling of cereal products.

- **Developing food composition data bases to better inform health professional and consumer**

For the B vitamins, there is a wide variation among vitamers and each vitamer might present very low bioavailability. However, the most interesting and comprehensive information for the consumer is to know the amount of biologically available vitamins present in the interested food products. Such reference information will facilitate food selection for interested consumers. From an industrial perspective (breeders, millers or industrial food processing), it might be more informative to properly measure individual vitamers. Thus, they will be able to monitor at each stage of the baking process the vitamin stability and the vitamers evolution in term of chemical forms. As shown earlier in the discussion, wheat varieties, milling process and cooking condition may favor the increase or the decrease of certain vitamers (e.g. niacin and vitamin B6). Without talking of bioavailability, food industry could focus on improving the amount of free vitamins which are mainly the form encountered in supplemented food. In addition, all the changes occurring during food processing could be registered in new food composition databases, and the important information relative to the amount of vitamins really absorbable could appear on nutritional labeling. Another option which could be considered is to develop a label called vitaminic index .Vitaminic index would be a number associated to cereal food products that would indicate the food effect on a person's absorption vitamin. The number could be ranged between 0 and 100, where 100 would mean that vitamins present in the food would be found only in free forms .The vitaminic index could be applied in the context of the amount of vitamin in the food that could be actually absorbed. Indeed, each cereal products could be ranked according to their fraction of bound forms of vitamin. Cereal products with the highest scores (i.e. highest amounts of bound forms compared to the free forms) for

these vitamins would have a minimum vitaminic index. On the contrary highest amounts of free forms compared to bound forms would have a maximum vitaminic index. Such project will give more clarity and transparency for the consumers on the nutritional value of cereal food products. During the discussion we have proposed reliable information about potential analytical methods which could be used to facilitate the measurement of individual vitamers. Because nowadays, there is an important demand from the consumers for nutritious cereal-based food products with minimal artificial additives, new discussion and consortium should emerge to allow all the actors of the wheat grain- product chain (breeders, millers, farmers, bakers and industrial food processing) to promote wheat food products with enhanced and legible nutritional values.

The variability in the level of wheat vitamins or phytochemicals depends on the wheat variety, environmental growing conditions and often interaction between these factors (Shewry et al., 2010; Shewry et al., 2011). Thus, the third objective of this thesis was to describe the genetic and environmental factors affecting the total contents of B and E vitamins, Lutein and β-sitosterol in a core collection of hexaploid wheat grown in two environments.

3. Evaluation of the core collection in term of agronomic and nutritional traits

3.1. General conclusion

The analysis described in our study have shown that :

- Genotype has a significant impact on the variations of vitamin contents, with lutein presenting the greatest range of variation among the vitamins analysed.
- The highest mean temperature over the growth period in Clermont-Ferrand have demonstrated a positive effect on the net synthesis of pantothenic acid and γ-tocopherol in bread wheat lines.
- The higher heritability of vitamins α-Tocopherol, β-γ-Tocotrienol, α-Tocotrienol, β-Sitosterol, lutein, thiamin, riboflavin and pantothenic acid indicated that increases in content may be possible by breeding.
- The contents of vitamins have not decreased with modern plant breeding.
- Varieties from the western parts of Europe might be potential reservoir of genotypes for improving the proportion of lipide-solublecompounds in wheat.
- The core collection has permitted to select lines which combined high levels of vitamins with high yield and good quality traits

3.2. Genetic and agronomic perspectives

- **Genetic levels**

The increasing global population places a clear priority on increasing food production. Furthermore, as mentioned by Shewry and Ward (2012), it is also important to consider the wider impacts of consumption of highly refined processed foods which are rich in fat and sugars and could be associated with major symptoms such as obesity, insulin resistance, hyperglycemia and hypertension when it is combined with a sedentary lifestyle. However, consumption of fruit, vegetable, wholegrain cereals or components present in those foods can protect against major chronic diseases (Fardet, 2010). In the present thesis, we have discussed about the extent of variation in vitamin contents and compositions due to variety and environment. We also showed that some bread wheat lines with good agronomic traits could be exploited to maximize health benefits. Nevertheless, our study focused on unbound bioactive components. It will be very informative to review in a near future the range of bound vitamins present in the bread wheat core collection. Because these components are not fully bioavailable, screening within the large collection for lines with low amounts of these bioactive components would be beneficial to develop new cereal products with improved nutritional value.

- **Agronomic levels**

At the agronomic level, the conventional agricultural practice may reduce the possibility of using the whole grains for nutritional purposes as most of the pesticide are accumulated in the external layers of the grains. In addition, the increases in the yield could cause decreases in the overall vitamins content because of the accumulation of starch to the detriment of the bran fraction. Thus, use of varieties with high TKW and high level of bioactive compounds grown under organic agriculture to reduce the pesticide contamination and increased the productivity of healthy whole-grains wheat should be further investigated.

In order to develop genetic markers to help breeders to select lines with enhanced nutritional value, it is necessary to identify the genes responsible for the traits, or at least to determine their precise locations on a genetic map (Shewry et al., 2012). With recent developments in statistics and high throughput genotyping, association analysis can now be applied to many different kinds of populations, such as genetic resource collections, for mapping regions responsible for the variation in many traits (Bordes et al., 2011). Thus, the last objective of this thesis was to scan the genome for new loci involved in the genetic control of wheat vitamins composition by association mapping in a core collection of 196 accessions genotyped with DArT, SSR and SNP markers on the wide genome.

4. Evaluation of the core collection for association analysis of nutritional traits

4.1. General conclusion and perspective

Linkage disequilibrium can be used for identifying associations between traits of interest and genetic markers. This study is the first report identifying chromosomal regions associated with complex vitamins trait using a bread wheat core collection built by maximizing the genetic diversity and phenotypic variability.

- Several regions involved in the genetic control of vitamin trait were identified.
- Several TFs of unknown function in grain were involved in this genetic control and detected under heat stress condition.
- Strong association between SAL gene candidate and α-Tocotrienol and thiamin content may reflect a strong accumulation of these vitamins in the aleurone layer of wheat grain.
- Strong correlations observed between the contents of thiamin, niacin and pyridoxine might be explained by the collocation association of some markers with those vitamins.
- Under drought stress environment, phenotypic variation of pantothenic trait was strongly related to the presence of the SAL-A putative gene in the long arm of chromosome 7A.

Results from this thesis show Dart and SSR markers significantly associated with the vitamin traits in chromosomal regions where genes or QTLs have been previously reported (Carotenoids), as well as significantly associated with markers in regions where QTLs have never been reported for those traits. The role of those regions will need to be further investigated. Several TFs of unknown functions expressed in grains were involved in the genetic control of the vitamins. Functional studies of these TFs are also needed in the future. Regions on chromosome 7A and 7B significantly associated with thiamin and pantothenic acid contents were in strong linkage disequilibrium with two candidate genes SALs. This may suggest that enzymes involved in the development of the aleurone layers affect those vitamins accumulation and that in order to protect the grain development from abiotic stress, SAL-A candidate gene play a key role in the synthesis of pantothenic acid. However, it remains to be determined if these genes are responsible for the associations that were detected. Fine mapping of the region in linkage disequilibrium with SAL-A and B candidate genes would help to identify more putative candidate genes. Finally, detecting QTLs or genes that influence bioavailable vitamin traits in wheat grain could help to increase the concentrations of bioavailable vitamins in wheats to develop wheat lines with enhanced nutritional values.

CONCLUSION

In conclusion, a great deal of perseverance and communication among the scientic community is needed, if we are to establish a sustainable form of agriculture in which the healthiness and safety of wheat and wheat based food products are the paramount considerations. During this thesis we have revealed different technologic, agronomic and genetic information which could be used to improve the nutritional composition in vitamins of wheat grain. An important attention should be given to the analytical methods used. In fact we have demonstrated the usefulness of developing new mass spectrometry tools to study on the composition of wheat grain or wheat grain products in active metabolite. However, it would be judicious in order to render this technology even more attractive and pertinent, to develop normalized mass spectrometry methods to quantify those active metabolites. Use of the standard methods will ensure that data labeling (as an example : the level of vitamin contents)for food products are reliable and enabling the direct comparison of the nutritional value of food products from different markets, companies or institutes. Furthermore, the standard methods have to be extremely specific on the actual bioactive compounds which have to be measured. As an example, niacin or vitamin B3 can be used in reference to all forms with vitamin activity (nicotinic acid, nicotinamide, NAD, NADP). In developing analytical methods which allow only the total vitamin B3 level to be quantifed, a lot of valuable information may be lost, and it may also creates a lot of misunderstanding when trying to decipher such data analysis. Because, global climate models predict changes in precipitation patterns with frequent episodes of drought, the other point which in my sense have to be discuss, concerns the impact of drought stress on the composition of wheat grain in vitamins. From our results, apart for pantothenic acid, pyridoxal and β-γ-tocopherol, in general heat stress causes a significant reduction in both grain size and level of vitamins. However, there is variation in these traits, with some varieties being able to retain either grain size, or vitamin levels after stress conditions. There is the potential to combine the positive effects of both vitamin content and grain size to improve heat stress tolerance in future varieties.

Finally, the discussion around the nutritional value of wheat based food products has to be more clear and transparent. There is a lot of solutions to improve the nutritional status of wheat grain, some of them have been exposed along this thesis, however semantic or complexity of the explanation used can hinder the development of this potential. As scientists, we have a duty to clarify as much as possible our finding to make it useful for all the actors of the wheat grain-product chain.

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Abbreviations

AM : Association mapping

APCI : Atmospheric pressure chemical ionization

CID : Collision-induced fragmentation

DAD : Diode-array detection

DArT : Diversity array technology

DM : Dry mass

ESI : Electrospray ionization

GPC : Grain protein content

GSPs : Grain storage proteins

GxE : Genotype environment interaction

HILIC : Hydrophilic interaction liquid chromatography

LC-MS/MS : Liquid chromatography/tandem mass spectrometry

LD : Linkage disequilibrium

LOQ : Limit of quantification

MRM : Multiple reaction monitoring

MTA : Marker-trait associations

NAD : Nicotinamide adenine dinucleaotide

NADP : Nicotinamide adenine dinucleotide phosphate

NIRS : Near infrared spectroscopy

QTL : Quantitative trait locus

SAL : Supernumerary aleurone layer

SIM : Selected ion monitoring

SNP : Single nucleotide polymorphism

SPA : Storage protein activator

SPE : Solid phase extraction

SSR : Simple sequence repeats

TFs : Transcription factors

TKW : Thousand-kernel weight

Tocols : tocopherols and tocotrienols

Vitamin B1 : Thiamin

Vitamin B12 : Cobalamin

Vitamin B2 : Riboflavin

Vitamin B3 : Nicotinic acid + Nicotinamide

Vitamin B5 : Pantothenic acid

Vitamin B6 : Pyridoxine + Pyridoxal+ Pyridoxamine

Vitamin B8 : Biotine

Vitamin B9 : Folate