AlgeaFood Phyto
for shaping your waistline, clinically tested
The intelligent way to make foods functional.

Indena may be a new name in the functional foods market. But we weren’t born yesterday. Far from it. For more than 90 years, we at Indena have been researching, developing and producing plant derived active principles. Natural solutions, whose safety and efficacy are guaranteed also by intensive research and rigorous testing. For many years, Indena products have been widely used and appreciated in the pharmaceutical, cosmetic and health food industries. We are already marketing a number of functional food ingredients. And more are on the way. To know more, indena.com.
For many years, Indena products have been widely used and appreciated for their derived active principles. Natural solutions, whose safety and efficacy we at Indena have been researching, developing and producing plant-based products for more than 90 years, are guaranteed also by intensive research and rigorous testing. In the pharmaceutical, cosmetic and health food industries, Indena may be a new name in the functional foods market. And more are on the way. To know more, visit indena.com.
Salt is an important nutrient if used in small doses: probably you are eating more sodium than your necessity. Moreover, sodium consumption is related with increase of blood pressure and cardiovascular diseases (CVD’s). Cardiovascular diseases (CVD’s) are a global problem that cause the death of over 17 million people only in 2008. More of 3 million of these deaths can be prevented and occurs in people aged less than 60 (1). Scientific evidence shows that simply reducing the population-wide sodium intake by 15% could prevent 8.5 million cardiovascular-related deaths worldwide over 10 years (2).

More recently, the case for decreasing salt intake has been reinforced by the European Food Safety Authority’s (EFSA) positive opinion, which concluded that there is a cause and effect relationship between high dietary sodium intake and increased blood pressure, and that a reduction in dietary sodium intake helps maintain normal blood pressure (ID 336) (3).

The WHO recommends a population salt intake of less than 5 grams per person per day to help prevent CVD. It is estimated that the current global levels are of 9–12 grams per day: for example Americans get most of their daily sodium -more than 75%- from processed and restaurant foods (4). To benefit the population and the economy, governments are pushing for population wide sodium reduction.

Salt is largely used because does not change the organoleptic characteristics of a food while is a taste enhancer, a preservation agent and is useful for food processing. These functions influence how, and if, sodium levels can be reduced in food (5). It may be a valuable solution to replace the common salt that can be found in processed foods with natural ingredients, such as seaweed for example. Ascophyllum nodosum can be a valuable ingredient to substitute part of the added sodium in processed food, poor in salt but having a natural richness of the flavour.

Therefore, in order to offer complete solutions we industrially tested AlgeaFood Phyto in different types of processed products like:

**SAUCE:** Mayonnaise with a little bit of mustard, reduction of salt 60%

**FOODS for VEGETARIANS:** Tofu with spinach and seaweed, salt reduction around 50%

**READY FOODS:** Tomato sauce with grouper, salt reduction 50%

**PROCESSED MEAT:** Paté of meat with Arctic seaweed, salt reduction 40%

No changes in the industrial parameters by using the Arctic phytocomplex, for sensorial evaluation all of these products were tested in a panel test with 106 panellists; all of them representative of general consumers’ population (age 25-55, women (55%), men (45%)) were individually interviewed for 30 minutes.
A powerful marine ingredient: application as a valuable salt replacer in industrial products

The test performed, in cooperation with a reliable partner, was with **four tastings**:
- **Blind** taste compared with the white for 2 products
- Explain to panellists the differences between the two products: seaweed to reduce salt and only salt:
- **Informed** taste of a product compared with the white
- **Placebo** taste of product with a wrong information regarding the presence of seaweeds or only salt

Here is an example, for tomato sauce with grouper for each taste: placebo effect works, because the “wrong” taste (the product that consumer believed with salt) achieves lower vote in all of three taste types.

Finally, there are a growing number of publications in scientific journals attempting to find **new ways to replace sodium chloride** without losing food flavour, and several of them are focusing on **brown seaweeds**. To mention just a few of them, it has been shown that brown seaweeds can also be successfully used in low-salt meat products (6) and in low salt seasonings (7), and the benefits of their use are not only reduced amounts of sodium used.

**Algea, The Arctic Company**
In Norway, beyond the Arctic Circle, in one of the purest habitats in the world, we have been harvesting and processing *Ascophyllum nodosum* with great passion for over 75 years. Because this seaweed undergoes strong climate stress due to being sometimes exposed to the sun and bad weather and other times immersed into freezing Arctic waters it developed active components of the highest quality and purity. We harvest *Ascophyllum nodosum* following a strict process to preserve its quality, with tools and technologies that are advanced in order to preserve the environment.

Processing takes place near harvesting areas to keep all the properties intact. It is how AlgeaFood Phyto is produced: a marine phytocomplex made with brown Arctic seaweed, rich in high quality natural active compounds than can help against fat accumulation and sodium intake.

The low content of sodium and a balanced intake of algal carotenoids, alginates, vitamins and minerals make AlgeaFood Phyto a great **alternative to kitchen salt**, helping the body better process fats and facilitating **weight control** and the achievement of natural well-being.

**References**
2. Keast, R. S.J. Hayes J. E., “Successful Sodium Reduction”, The World of Food Ingredients, September 2011
Very recently, Golomb and coworkers published a paper with the meaningful title “A Fat to Forget” in which they show evidence that the consumption of dietary trans fatty acids (TFAs) predicted declines in memory, and that memory became worse with increased intake of trans fats [1]. This is only one of the recent additions to the list of evidence of the harmful effects of TFAs on human health. However, while for years TFAs have been a well-recognised risk factor for the development of cardiovascular diseases and associated with all-cause mortality (2), products with high TFA levels can still be found in some markets. An extensive market basket investigation of biscuits in 20 European countries performed by Stender and co-workers showed that TFA levels in foods in Eastern Europe are much higher than in Western Europe (3). These results suggest that some population groups might still be consuming TFAs in amounts that increase their health risks.

TFAs are isomers of unsaturated fatty acids which are not synthesised by the human body and not required in the diet. Their biggest source in the diet is processed foods which contain partially hydrogenated oils (PHOs). These are used to produce semi-liquid and solid fats that can be included when producing foods such as margarine, shortenings and biscuits with a long shelf life. Such products can constitute up to 60% of TFAs (3). PHOs can also withstand repeated heating and are therefore used for frying. For these reasons, as well as consumers’ positive perception of vegetable fats (compared to animal fats), PHOs became very popular in the middle of last century.

The first safety concerns with TFAs present in PHOs emerged in the 1970s and suggested an increased risk of coronary heart disease. Based on strong scientific evidence, it was concluded that TFA intake should be as low as possible within the context of a nutritionally adequate diet [4]. Responsible food manufacturers reformulated food products and stopped using PHOs, while policymakers started to regulate this area. Various food policies are being used in different countries to limit dietary intake of TFAs, for example mandatory labelling of the presence of PHOs (currently in use in the EU), labelling of the content of TFAs, and the most restrictive policy – regulatory limits on TFA content in foods.

In Canada and the USA, compulsory labelling of the amount of TFAs in foods was introduced in 2005 and 2006, respectively. In 2013, the US FDA published a preliminary report stating that PHOs were no longer Generally Regarded as Safe (GRAS) and, very recently, in June 2015, it was decided that PHOs will no longer be allowed in foods (5). The food industry has a three-year compliance period to phase out industrial TFAs.

In the EU, mandatory labelling of PHOs on pre-packed food products was introduced in the 1990s. Currently, PHOs must be labelled on ingredient lists as “partially hydrogenated fat/oil”, but there are no general EU limits on TFA levels in foods. Most members currently rely on food producers to voluntarily reduce TFAs. Denmark was the first EU country to introduce legislative limits on TFAs in 2004, prohibiting the sale of foods containing more than 2 g of industrial TFAs per 100 g of fats. Similar rules were introduced later in Austria (2009) and in Hungary (2014).

The acceptance of Regulation (EU) No 1169/2011 on the provision of food information to consumers (6) provided an opportunity to harmonise this issue on the EU level. According to this regulation, the EC shall prepare a report on the presence of TFAs in foods and in the overall diet of the EU population, including a proposal to provide information about TFAs to consumers or restrictions on their use. The EC shall accompany this report with a legislative proposal, if appropriate (6).

While such a report should have been published before December 2014 it has been delayed and its publication is expected these days. The EC has been under strong public pressure to resolve this issue as soon as possible. A number of organisations, including the European Society of Cardiology, have called on European policymakers to ban the use of industrial TFAs. It seems that member states are in favour of such a decision, and there are no opponents publicly defending the use of PHOs in foods. It seems like an excellent opportunity to permanently forget PHOs as a food ingredient also in the EU. While this would be a significantly delayed decision, it would be a very important one. Because several countries are looking closely at food policy decisions in the EU, such a decision would also have a strong global message.

REFERENCES
5. US FDA. 2015. Final Determination Regarding Partially Hydrogenated Oils (Removing Trans Fat)
6. Regulation (EU) No 1169/2011 on the provision of food information to consumers
Superior minerals for infant formula

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Improved taste of final product

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From nature to ingredients®
INSECTS AS FOOD AND FEED: WHAT ARE THE RISKS?

Interest is growing in the potential benefits of using insects in food and animal feed, but what would be the risks from production, processing and consumption of this alternative source of protein?

EFSA has addressed this question with a risk profile that identifies the potential biological and chemical hazards as well as allergenicity and environmental hazards associated with the use of farmed insects as food and feed. The Scientific Opinion also compares these potential hazards with those associated with mainstream sources of animal protein.

The possible presence of biological and chemical hazards in food and feed products derived from insects would depend on the production methods, what the insects are fed on (substrate), the lifecycle stage at which the insects are harvested, the insect species, as well as the methods used for further processing. EFSA’s scientific experts say EFSA concludes that when non-processed insects are fed with currently permitted feed materials, the potential occurrence of microbiological hazards is expected to be similar to that associated with other non-processed sources of protein. There are limited data available on the transfer of chemical contaminants from different types of substrate to the insects themselves.

The occurrence of prions – abnormal proteins that can cause diseases such as Bovine Spongiform Encephalopathy (BSE) in cattle and Creutzfeldt-Jakob Disease in humans – is expected to be equal or lower if the substrate does not include protein derived from humans (manure) or ruminants.

The Scientific Opinion also considers the possible hazards associated with other types of substrate, such as kitchen waste, and animal manure.

The environmental risk of insect farming is also expected to be comparable to other animal production systems. Existing waste management strategies should be applicable for disposing of waste from insect production.

EFSA’s opinion is based on data from peer-reviewed scientific literature, assessments performed by Member States and information provided by relevant stakeholders.

Background

Insects represent a niche food market in the EU, with several Member States reporting occasional human consumption. Nonetheless, the use of insects as a source of food and feed potentially has important environmental, economic and food security benefits. The insect species reported to have the greatest potential for use as food and/or feed in the EU include houseflies, mealworms, crickets and silkworms.

A number of organisations – including the Food and Agricultural Organisation of the United Nations (FAO) – have studied the possibility of using insects for food and feed, and three EU Member States – Belgium, France and the Netherlands – have performed risk assessments related to insects as food or feed.

The European Commission is currently co-financing a research project to explore the feasibility of using insect protein for feed. The Commission is also considering how to develop policy in the areas of novel foods and animal feed to reflect the potential use of insects as food and feed. EFSA’s Scientific Opinion was requested to support this work.

SCIENTIFIC OPINION ON THROMBIN FROM CATTLE AND PIG’S BLOOD

Following a request from the European Commission, the EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF Panel) was asked to deliver a scientific opinion on the food enzyme thrombin (EC 3.4.21.5) obtained from blood plasma of cattle and pigs.

The food enzyme is obtained from cattle and pig’s blood that is fit for human consumption and is processed hygienically. The food enzyme is used together with added fibrinogen; the thrombin catalyses the transformation of fibrinogen to fibrin, which interacts with collagen, enabling the binding of meat/fish pieces resulting in meat preparations, meat products and fishery products.

The typical use and the recommended maximum use levels of the food enzyme have been provided.

Dietary exposure to thrombin from its use as a food enzyme was estimated using the EFSA Comprehensive European Food Consumption Database. The estimated mean and 95th percentile exposure across five population groups ranged from 3 to 12 µg TOS/kg body weight/day and from 6 to 24 µg TOS/kg bw/day, respectively.

The food enzyme has been characterised by determining the temperature and pH optima and the thermostability. Its composition is characterised by measuring the enzyme activity, content of protein and total organic solids.

As the food enzyme is derived from edible parts of animals, no toxicological tests are required.

Considering the origin of the food enzyme, the CEF Panel considers that the likelihood of a food allergic reaction to this thrombin is low and, therefore, it does not give rise to safety concerns.

Based on the origin of the food enzyme from edible parts of animals, the manufacturing process, and the compositional and biochemical data provided, the Panel concluded that this food enzyme does not give rise to safety concerns under the intended conditions of use.

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The new EZ-Test SX/LX series are powerful Texture Analyzers for applications in food and electronics industries as well as in quality control of plastics and rubber. In addition to the long and the short column versions, a high-speed model is available.

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Nutritional genomics and disease prevention: a case for colorectal cancer

Abstract
Within a regular diet, there are many bioactive compounds that have been shown to exhibit chemopreventative properties. In many cases, this is due to their interactions with the genome, or, moreover, the epigenome. Nutritional genomics is the field of study that aims to elucidate the mechanisms and downstream effects that said bioactive compounds employ with respect to disease. Of the chronic ailments that can be modulated by the diet in such a way, cancer prevention is the most heavily studied, particularly colorectal cancer (CRC). This interest is due to the increased exposure and susceptibility for the colonic microenvironment to be modulated by small molecules in the diet. Understanding these mechanisms and implications in cancer prevention is vital for the future of dietetics and personalized medicine.

*The following case study is entirely theoretical. Its purpose is to illustrate the potential understanding of how dietary constituents can impact the onset and progression of cancer. All patient information in the case study is fictional. Further research is needed for such medical and dietetic advances.

CASE STUDY
Patient 1 is a fifty-year-old male presented with blood in stool and abdominal pain. A colonoscopy showed an ulcerative non-metastatic tumor. He subsequently underwent surgical extirpation of the tumor. The patient exhibits central obesity with elevated cholesterol, hypertension, heart disease, and diabetes with neuropathy. He has a history of alcohol consumption and smoking. The family history revealed that both his father and uncle died of colorectal cancer and his mother was obese and possessed type II diabetes.

INTRODUCTION
Colorectal cancer (CRC) is the third most common cancer in men and second in women worldwide (1). Recently, Dr. Bert Vogelstein published data that showed a linear correlation with the number of cell divisions and cancer incidence within specific tissues (2). As colorectal cells have one of the highest turnover rates, incidence of CRC is also high (2). This is due to the increased rate of mutations that occur in highly proliferating tissues (2). Additionally, colorectal tissues are highly susceptible to environmental exposure.

In addition to being one of the most common cancers, it is also one of the most easily preventable (3). A large part of this prevention hypothesis is due to the exposure of the colon to small molecules from the diet, many of which have properties that allow them to modulate the molecular environment of the cells (3). Prevention is largely achieved by controlling unwanted DNA adduct formation and altered epigenetic changes within the microenvironment of the colon.

With global CRC rates on the rise, researchers are looking for new forms of prevention or treatment that are less expensive and more readily available through the diet (1). The regulation of gene expression by small molecules in the diet has recently attracted a great deal of research. This field of study is termed nutritional genomics. Nutritional genomics aims to understand the association between the genome and bioactive compounds in the diet, and their impact on health and disease.

NUTRITIONAL GENOMICS, MECHANISMS
Nutritional genomics is defined as the study by which dietary constituents can modulate gene expression and how genetic variation can influence an individual’s response to dietary constituents (4). In most applications, the downstream impact is the prevention and control of disease, while in some cases the opposite is possible.

Over the past decade, this field of study has gained interest among researchers, namely those interested in CRC (5). A few of the mechanisms and dietary interactions related in genetic variation and gene expression are discussed following.
Folate
One example of a dietary constituent that plays multiple roles in the realm of nutritional genomics is folate. Folate is widely known as the B vitamin involved in proper neural tube development, and is now added to processed cereal grains for fortification in many countries [6]. Additionally, it is naturally found in green leafy vegetables, such as spinach. However, the over supplementation of folate is beginning to gain attention as serum folate concentrations have increased over 100% since mandatory fortification began in the United States [7]. Notably, the developmental genes that are involved in normative neural tube development (and others) can also act as oncogenes when activated at improper time points within an individual’s lifecycle. This makes the role of folate in the regulation of these genes extra important in cancer progression.

Once in the body, dietary folate equivalents are converted to 5-methyltetrahydrofolate (MTHF), which is involved in the conversion of homocysteine to methionine. The methyl groups that are donated from methionine and choline are then used to produce S-adenosylmethionine (SAM). SAM is considered to be the universal methyl donor, and is involved in the action of DNA methyltransferases (DNMTs) [8]. DNMTs transfer methyl groups from SAM to cytosine residues in CpG rich promoter regions of DNA. Hypermethylation can cause genomic silencing, which is key for tumor suppressor genes in cancers. The involvement of folate in this pathway is important, as supraphysiologic concentrations of folate could lead to increased aberrant methylation patterns, and therefore increased susceptibility to cancers, such as CRC.

Additionally, folate is involved in thymidylate synthesis, which is the rate-limiting step in DNA synthesis [6]. Impairment of thymidylate synthase, the enzyme that catalyzes the synthesis of deoxythymidine monophosphate (dTMP) from deoxyuridine monophosphate (dUMP), can lead to the misincorporation of uracil into DNA [9,10]. This misincorporation can cause point mutations in the DNA, and, with increasing driver mutations, susceptibility to carcinogenesis increases. This is important in determining the proper dose of folate for chemoprevention, as too much folate leads to increased DNA synthesis and therefore increased cellular proliferation [9,10]. Importantly, it has been shown that high folate intake is protective in most individuals, the exception being those with preexisting neoplastic lesions [9, 10].

Methyl-tetrahydrofolate reductase (MTHFR) is another important enzyme involved in folate metabolism, and is key in DNA synthesis and methylation [6, 8, 10]. A common mutation, C677T, results in reduced activity of MTHFR (30% reduction in heterozygotes, 65% reduction in TT homozygotes) [10, 11]. The homozygous TT genotype is associated with increased CRC incidence and global DNA hypomethylation [6, 10, 11]. Realistically, serum folate concentrations require careful monitoring in at risk individuals, in order to offset the progression of CRC.

**CASE STUDY**
**Impact on Patient 1:** Prior to diagnosis, patient one had been taking 1 mg of folic acid equivalents in supplemental form daily. This dose is 1.5 times greater than the recommended daily allowance of folate, and has shown to increase CRC progression in a few studies [9,10]. As patient 1 likely possessed a preneoplastic lesion, this supraphysiologic dose of folate could have promoted tumorigenesis.

Methylation
Methyl groups are single carbon units that can be transferred to small molecules, proteins, and DNA via a variety of mechanisms, primarily by DNMTs. The methylation of DNA occurs in cytosine rich regions, called CpG islands. When CpG islands are in abundance in the promoter regions of DNA, and can therefore become methylated, the gene in question is usually silenced [5,12]. Hypermethylation can result in the aberrant silencing of tumor suppressor genes, where hypomethylation can result in the activation of oncogenes. Both of these scenarios are undesirable. However, the opposite can also be true, leading to a more positive outcome.

Caffeic acid phenethyl ester
Caffeic acid, a natural polyphenol found in coffee, is recognized for its antioxidative and anti-inflammatory properties. Recent studies have shown that caffeic acid inhibits the proliferation of cancer cells and may be an anti-cancer agent itself [13]. Caffeic acid phenethyl ester (CAPE), a derivative of caffeic acid, has also been shown to have anticancer properties. Two proteins involved in tumorigenesis, PSAT1 and PSMA1, were both down-regulated after CAPE treatment [14]. Several studies have indicated that CAPE induces apoptosis in liver, oral, and colon cancer cells, in many models. It has also been shown to inhibit the development of azoxymethane-induced aberrant crypts in the colon of rats [14]. These findings show increasing promise that caffeic acid may be used as a chemopreventive agent against certain forms of cancer, including CRC.

Epigallocatechin-3-gallate
A major phytochemical in green tea, epigallocatechin-3-gallate (EGCG), has long been known for its health benefits [15]. This is especially true in cancer prevention, through various mechanisms including decreasing global DNA methylation levels, reactivating estrogen receptor-alpha expression in breast cancer, or the demethylation of the Wnt oncogene promoter in lung cells [4]. CRC is a recent addition to the list of cancers that EGCGs may have protective effects on. A study conducted by Chen et al. indicated that after human CRC cells were treated with EGCGs for 36 hours, EGCGs caused mitochondrial damage and induced apoptosis triggered by oxidative stress [16]. Another study demonstrated that EGCGs from green tea have the ability to inhibit DNMT activity and reanimate methylation-silenced genes in cancer cells [17]. This reactivation of methylation-silenced genes through the usage of EGCGs was demonstrated in human CRC, esophageal cancer, and prostate cancer cell lines.

EGCG have also been shown to have anticancer activity by inducing cellular apoptosis, specifically for patients with CRC. When sodium butyrate (NaB), a naturally occurring non-toxic compound produced in the colon, is combined with EGCG for treatment, apoptosis and cell cycle arrest in various CRC cell lines occurs. In addition, it was found that survivin, an antiapoptotic protein that is usually over-
expressed in CRC, was down-regulated in the CRC cells when exposed to both NaB and EGCG (18). This combined treatment provides a novel approach to combating CRC with naturally occurring compounds at lower than conventional doses.

**CASE STUDY**

**Impact on Patient 1:** Patient 1 recently disclosed his TAS2R38 genotype, which revealed that he is considered to be a “superaster” (19). This means that patient 1 is highly sensitive to bitter compounds, which are present in high concentrations in coffee, tea, and certain vegetables. Consequently, patient 1 typically turns to soda instead of coffee or tea for caffeine. This choice provides patient 1 with minimal CAPE and EGCG, both of which have shown to be beneficial in CRC prevention. Had patient 1 ingested such compounds, apoptosis of aberrant cells could have been encouraged, therefore leading to decreased carcinogenesis.

**POST-TRANSLATIONAL MODIFICATIONS**

Post-translational modifications regulate the balance between the survival and death of cells, and can be modulated through the diet. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) induced post-translational modifications have been recognized to play a role in triggering apoptosis and may be a promising method of targeting cancer cells (20–22). Autophagy, which is a cellular process of turnover for useless proteins and organelles, can be considered a temporary and reversible path to cell death. It has been shown that RNS induces autophagy through suppression of the mTORC1 pathway, decreasing cell viability in MCF-7 breast cancer cell line (20). Recent studies have indicated that many cancers, including breast and colorectal cancers, are initiated and maintained by cancer stem cells (CSC), which can initiate tumorigenesis. CSC may also contribute to tumor recurrence, metastasis, and treatment resistance in some cancers. CSC can be counteracted through aberrant N-linked glycosylation of cell surface receptors. This action has been widely implicated in regulating tumorigenicity and tumor progression, affecting CSC self-renewal (21). N-linked glycosylation of cell surface receptors can be altered through changes in expression of glycan-modifying enzymes (21). Altered expression of glycans-modifying enzymes is a result of aberrant expression of sialic acids that cap these glycan chains. An increased intake of the non-human Sia N-glycolylneuraminic acid (Neu5Gc), a natural component found in milk and red meat, results in metabolic-glycoengineering of human carcinomas (22).

**Sulforaphane**

Sulforaphane is a bioactive metabolite of glucoraphanin, a glucosinolate found in cruciferous vegetables such as broccoli and Brussels sprouts. It is well understood that glucoraphanin induces the activity of phase II detoxification enzymes, and the metabolite then modulates HDAC activity and histone coding, primarily by inhibition (27). HDAC inhibitors, such as sulforaphane, cause increased acetylation of histones, which opens up the chromatin in those regions such that those genes are more accessible to transcriptional machinery (28). Increased acetylation allows for the activation of epigenetically silenced genes, which, in cancer are commonly tumor suppressors and other regulators of the cell cycle (28).

In an *in vitro* study by Rajendran et al., colon cancer cells were treated with sulforaphane and analyzed via a number of methods (27). They found that while the vehicle-treated control cells had a dramatic increase in cell viability and cell count, the sulforaphane treated cells showed no change in viability and the cell count remained constant. Additionally, HDAC activity in the sulforaphane treated cells did not change, compared to the control cells where HDAC activity was increased. Moreover, a significant reduction in class I HDAC protein expression was found in the cells treated with sulforaphane, especially in HDAC3. When sulforaphane

While the case study highlights that antioxidants can be detrimental to a person when oversupplemented, antioxidants also have the ability to enhance cancer-fighting mechanisms. ROS and RNS can also inhibit cancer-fighting mechanisms in the body, and antioxidants are able to extinguish these free radicals, allowing the cancer-fighting mechanisms to continue (24). For this reason, a balance between antioxidants and free radicals (such as ROS and RNS) is necessary for proper body maintenance. Without this balance, undesirable outcomes can accrue.

**Histone coding**

A more specific form of post-translational modification is through histone coding. Histones are small proteins that octamericize and are then wound by DNA. These complexes form nucleosomes, which are the basic components of chromatin. The “openness” of the chromatin determines the activity of transcription of the genes present in that area, with more accessible chromatin being considered “open” (25,26). The state of accessibility is determined by the small functional groups that are attached to lysine residues on the histone tails. Acetylation, methylation, phosphorylation, and ubiquitination are a few examples of the types of reactions carried out that add the respective functional groups to histones, the most predominantly studied being acetylation (27,28). These reactions are catalyzed by histone acetylases (HATs) and histone deacetylases (HDACs), which add and remove acetyl groups, respectively. Increased acetylation leads to more accessible chromatin, and therefore increased transcription of the genes in those regions (29).
was removed from the treatment, there was a reversal in HDAC protein expression and general increase in HDAC activity (27). The downstream effect is that histones remain acetylated, and therefore the genes in such regions can be epigenetically reactivated (27,28). This is important in cancer, as many tumor suppressors are silenced in cancer cells. Activating these genes allows for increased transcription, and therefore induction of apoptosis and cell differentiation, and the reduction of proliferation by cell cycle regulation (25–29).

**CASE STUDY**

**Impact on Patient 1:** As previously discussed, patient 1 is considered a supertaster. As bitter compounds, such as glucosinolates, are particularly potent to patient 1, he avoids cruciferous vegetables. This choice minimizes his exposure to sulforaphane, the active metabolite of a glucosinolate. This avoidance of cruciferous vegetables may have diminished patient 1’s susceptibility to CRC prevention.

**Grape seed extract**

Recently, the impact of grape seed extract (GSE) on miRNA expression and its downstream effects on signaling pathways in CRC has been under investigation. In a novel study by Derry et al., the chemopreventive effects of GSE regulated miRNAs were studied in a mouse model (31). Mice were injected with a chemical carcinogen, azoxymethane (AOM), in order to induce CRC, and fed diets containing various amounts of GSE, in hopes of establishing a dose-response relationship. The five groups were later evaluated for miRNA and cytokine expression. They found that GSE altered expression of cytokines/interleukins involved in proliferation and apoptosis (31). Additionally, they found that the AOM + 0.5% GSE group had very large changes in miRNA expression for multiple miRNAs when compared to the AOM only group (31). Importantly, the upregulated miRNAs, including miR-let7a and miR-20a, are regulators of cell cycle regulatory proteins (31–33). The increased expression of these miRNAs provides a survival advantage for those with CRC (32). The downregulated miRNAs, including miR-135b and miR-21, are responsible for the regulation of tumor and metastasis suppressing genes (31–33). Decreased expression of these miRNAs results in increased tumor and metastasis suppression (33).

**CASE STUDY**

**Impact on Patient 1:** Patient 1’s most recent food frequency questionnaire exposes his high consumption of grapes and red wine. Therefore, it can be hypothesized that patient 1’s tumor suppressing miRNAs are upregulated, while his oncogenic miRNA levels are downregulated. This could contribute to delayed onset and decreased progression of carcinogenesis.

**Table 1.** Dietary bioactive compounds, their genomic mechanisms, and downstream effects (4).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>Food source</th>
<th>Mechanism &amp; downstream effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caffeic acid phenethyl ester</td>
<td>[13, 14]</td>
<td>Coffee</td>
<td>Antioxidative and anti-proliferative properties, pro-apoptotic</td>
</tr>
<tr>
<td>Curcumin (34–37)</td>
<td></td>
<td>Turmeric, curry</td>
<td>Anti-inflammatory, antioxidant, anti-angiogenic and anti-cancer properties, inhibits DNMT activity</td>
</tr>
<tr>
<td>Epi-gallocatechin-3-gallate (41-18)</td>
<td></td>
<td>Green tea</td>
<td>Inhibits DNA methyltransferases, acts as histone modifiers</td>
</tr>
<tr>
<td>Folate (6,1–11)</td>
<td></td>
<td>Leafy greens, fortified grains</td>
<td>Methyl donor, influences DNA synthesis, genotype component</td>
</tr>
<tr>
<td>Genistein (38, 39)</td>
<td></td>
<td>Soybeans, fava beans, kidney</td>
<td>Regulate gene transcription by affecting histone acetylation and/or DNA methylation</td>
</tr>
<tr>
<td>Grape seed extract (31, 40, 41)</td>
<td>Many compounds</td>
<td>Grape seeds</td>
<td>Regulator of miRNA expression, which silence or activate oncogenic or tumor suppressing miRNAs, respectively</td>
</tr>
<tr>
<td>Isothiocyanates (42, 43)</td>
<td>Cruciferous vegetables (broccoli, cabbage, Brussels sprouts)</td>
<td>Proapoptotic and antiangiogenic properties, inhibits cancer cell growth</td>
<td></td>
</tr>
<tr>
<td>Reactive oxygen/nitrogen species (20-22)</td>
<td>Many compounds</td>
<td>Cured meats, ubiquitous in nature</td>
<td>Regulation of protein degradation by the formation of protein and DNA adducts</td>
</tr>
<tr>
<td>Resveratrol (44-45)</td>
<td>Grape skins, cranberries, blueberries</td>
<td>Involved in signaling pathways that control cell growth/division, apoptosis, angiogenesis, and metastasis</td>
<td></td>
</tr>
<tr>
<td>Selenium (47–49)</td>
<td>Brazil nuts, chicken, beef</td>
<td>Essential mineral in diet, involved in antioxidant, proapoptotic, DNA repair and anticancer pathways. Can restore the expression of antitumor genes silenced by hypermethylation</td>
<td></td>
</tr>
<tr>
<td>Sulforaphane (25–29)</td>
<td>Cruciferous vegetables (broccoli, cabbage, Brussels sprouts)</td>
<td>Regulates gene expression by inhibiting histone deacetylase activity, therefore altering histone acetylation and gene expression</td>
<td></td>
</tr>
</tbody>
</table>

**OTHER CHRONIC ALIMENTS IMPACT CARCINOGENESIS**

In addition to the previously discussed mechanisms, targeting the upstream predispositions for CRC are also important. Obesity and diabetes compose two of the most important extraneous factors that are often attributed to CRC (50).

**Obesity**

Obesity and its many comorbidities have become highly prevalent in the past 4 decades, affecting both young and old (51). Research suggests that obesity is
a transgenerational disease, and that treatment must encompass strategies for mitigating obesity in future generations. Both maternal and paternal obesity are been linked with body weight of future generations. For mothers, diets high in fat during gestation selects for an obesogenic phenotype in fetuses thereby increasing the chances of obesity and metabolic syndrome in offspring (52). In a study conducted by Soubry et al., paternal obesity was found to be associated with the Insulin-like Growth Factor 2 (IGF2) gene hypomethylation in newborns, indicating that paternal weight does affect the health of future offspring (53).

**Diabetes**

Obesity is a strong predictor of diabetes in a person. More than 90% of diabetes cases are accounted for by Type II Diabetes [T2DM], which is largely modulated by lifestyle choices. A study by Reymann et al. indicated that severe vitamin D deficiency in childhood obesity is associated with lower insulin sensitivity and higher levels of inflammatory mediators (54). However, another study revealed that when this deficiency is corrected, in some cases, so was the patient’s insulin sensitivity problems (55). The above example indicates that it is not impossible to counteract predisposed maternal and paternal traits through dietary choices.

**CONCLUSION**

There are several important factors that one should understand before diving head first into the field of nutritional genomics. It is a new and emerging field, and has been discussed here as a small piece of a larger picture. Patient 1 may have benefited from increased caffeic acid, sulforaphane, and vitamin D intake to ameliorate some of his symptoms and delay cancer progression, but this treatment plan does not apply to everyone. It is imperative that we understand that an individual’s genome and epigenome is unique. While there are some similarities, if we choose to use food as a form of preventative medicine, ultimately, every patient will have a treatment plan specifically tailored to them. The field of nutritional genomics is rapidly expanding, as mechanisms are being elucidated at an increasing rate. In order for medicine and dietetics practice to continue advancing, changes in scientific knowledge need to be embraced. We have now seen that epigenetics may have profound effects on diet, lifestyle, and overall health. What an individual eats is driven by many factors; such as culture, taste and smell, affordability, and psychological or emotional factors. We know that taste can be genetically embraced. We have now seen that epigenetics may ameliorate some of his symptoms and delay cancer progression, but this treatment plan does not apply to everyone. It is imperative that we understand that an individual’s genome and epigenome is unique. While there are some similarities, if we choose to use food as a form of preventative medicine, ultimately, every patient will have a treatment plan specifically tailored to them. The field of nutritional genomics is rapidly expanding, as mechanisms are being elucidated at an increasing rate. In order for medicine and dietetics practice to continue advancing, changes in scientific knowledge need to be embraced. 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Scientific opinion on the safety of the extension of use of steviol glycosides (E 960) as a food additive

KEYWORDS: food additive, steviol glycosides, E 960, sweetener, extension of use.

Following a request from the European Commission to take into account an extension of use of steviol glycosides (E 960) as food sweeteners, a revised exposure assessment for five population groups was carried out based on the maximum permitted levels (MPLs) authorised in Annex II to Regulation (EC) No 1333/2008 and the extension of use at the levels proposed by the applicant for the food category 14.1.5 Coffee, tea, herbal and fruit infusions, chicory; tea, herbal and fruit infusions and chicory extracts; tea, plant fruit and cereal preparations for infusions, as well as mixes and instant mixes of these products (sub-category 14.1.5.2 Other).


The present exposure estimates of steviol glycosides (E 960) are based on the MPLs of use currently authorised in Annex II to Regulation (EC) No 1333/2008 and take into consideration the maximum levels as proposed by the applicant for an extension of use in tea, coffee, herbal infusion beverages, instant coffee and instant cappuccino products up to 29 mg/L of steviol equivalents, rather than 10 mg/L, as assessed in the previous 2014 EFSA opinion (EFSA ANS Panel, 2014).

The individual raw consumption data for five population groups (toddlers, children, adolescents, adults and the elderly) from the EFSA Comprehensive European Food Consumption Database were used for the calculation of the revised exposure assessment of steviol glycosides (E 960).

According to this requested extension of use, the impact on dietary intake appears to be negligible in comparison with the revised estimate of 2014. The Panel noted that, overall, the mean exposure estimates remain below the ADI of 4 mg/kg bw per day for all population groups, with the exception of toddlers (in one country) at the upper range of the high-level exposure estimates (95th percentile: 4.3 mg/kg bw per day), which remains above the ADI.

The Panel concluded that dietary exposure to steviol glycosides (E 960) is similar to the exposure estimated in 2014 and therefore does not change the outcome of the safety assessment (EFSA ANS Panel, 2010, 2014).
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The effects of bilberries, blackcurrants and their constituent anthocyanins on heart health in humans

KEYWORDS: Berries, anthocyanins, polyphenols, supplementation, cardiovascular disease, atherosclerosis.

Abstract
Berries have a long history of use in humans, as they have been shown to have numerous positive health effects. In this review the focus is on the effects of bilberries and blackcurrants and their purified extracts containing higher levels of anthocyanins on human heart health. Anthocyanins have an effect on several different areas of cardiovascular health, including improvement of endothelial function, anti-oxidative effects, anti-inflammatory effects and the normalization of HDL and LDL lipoproteins in the blood. Purified anthocyanin supplements derived from bilberry and blackcurrant show positive effects against all these cardiovascular disease targets.

INTRODUCTION
Berries have been consumed for many centuries because of their beneficial effects for vitality, prevention of viral infections, eye health, bladder and kidney health, and gastro-intestinal health (1). The interest in dietary supplementation with berries has increased in recent times because of the recognition of their beneficial effects on cardiovascular disease, cancer prevention, neurodegenerative diseases, aging skin protection, inflammation-related diseases, type 2 diabetes and metabolic syndrome (2, 3).

Bilberries and blackcurrants are an important source of micro- and macronutrients. Their beneficial health effects have been associated with the high levels of bioactive substances they contain, such as phenolic acids, flavonoids and especially anthocyanins. Anthocyanins are the largest group of water-soluble pigments in the plant kingdom and are responsible for the red, blue and purple colors of many fruits and vegetables. Products with high anthocyanin content include berries, red grapes, red cabbage, eggplant and the black variants of soybean and rice. Anthocyanins are classed as flavonoids, part of the (poly)phenolic group of natural products, and are usually found in the form of flavylium cations. Anthocyanins are classified according to the number and location of the hydroxy and methoxy groups attached to the flavan nucleus, and by the nature of the attached mono-, di- or tri-saccharides, which can contain glucose, galactose, arabinose, rhamnose and xylose groups (4,5). The six main anthocyanidins found in nature are pelargonidin, cyanidin, delphinidin, peonidin, petunidin and malvidin, with cyanidin as the most widely occurring (Figure 1).

The average anthocyanin content of bilberry is around 430 mg and that of blackcurrant is around 270 mg per 100 g of edible fruit (6). Bilberries contain a wide variety of at least 16 different anthocyanins; the main ones are delphinidin-3-glucoside (D3G), delphinidin-3-galactoside (D3Gal), cyanidin-3-glucoside (C3G), cyanidin-3-galactoside (C3G), delphinidin-3-glucoside (D3G), delphinidin-3-galactoside (D3Gal), cyanidin-3-glucoside (C3G), cyanidin-3-galactoside (C3G).
endothelial function

Endothelial cells line the blood vessels and play an important role in vascular health. Impaired endothelial function leads to a lower ability of the blood vessel to dilate because of the lower availability of nitric oxide (NO) (a vasodilator), and other vasoactive molecules such as endothelin-1 and prostacyclin. Nitric oxide synthase (NOS) is present in the endothelial cell, and converts arginine into NO. This diffuses into the vascular smooth muscle cells and activates guanylyl cyclase (GC), leading to the production of cGMP. This second messenger activates a chemical cascade leading to vasodilation (see Figure 2).

Several randomized placebo-controlled trials have been performed to determine the effect of blackcurrant juice and purified anthocyanins from bilberry and blackcurrant on endothelial function. They show that a short-term blackcurrant juice consumption in healthy individuals (single consumption of 250 mL 20% blackcurrant juice, providing 50.5 mg total cyanidin and delphinidin) does not have a significant effect on endothelial dependent or endothelial independent vascular reactivity, and on biomarkers of endothelial function (plasma nitrite, nitrate, ICAM-1 and VCAM-1) (13). A blackcurrant juice supplementation study in healthy volunteers with a habitual consumption of less than 2 portions of fruit and vegetables per day (4 x 250 mL blackcurrant juice providing 143 mg anthocyanins per day for 6 weeks) found a significant increase in FMD. These effects are attributed to the improved endothelial function, as the endothelium-independent glyceryl trinitrate-induced dilation was not affected. Oxidative stress, measured as F2-isoprostanes, was significantly lowered after supplementation (14). As the long-term blackcurrant supplementation study does show an improvement in endothelial function, the lack of effect in the earlier trial is most likely due to low amount of active constituents consumed.

It is not only blackcurrant juice which shows positive effects on endothelial function. Supplementation studies using purified anthocyanins from bilberry and blackcurrant in hypercholesterolemic subjects (320 mg anthocyanins per day for 12 weeks) showed significantly increased FMD and cGMP levels. The cGMP levels and FMD were found to influence by anthocyanin supplementation. Also on exposure to the NO-cGMP inhibitor L-N-monomethyl arginine (L-NMMMA), the beneficial effects on FMD were abolished. This has been confirmed in a rat aortic ring model in the same study. The levels of VCAM-1 were also significantly decreased (15). A similar study using the same anthocyanins, the same dosage and type of subjects over 24 weeks also found that the endothelial adhesion factor VCAM-1 was significantly decreased in plasma. It was confirmed in a porcine arterial endothelial cell line that the anthocyanins C3G and D3G are most likely to be responsible for the significant reduction of VCAM-1 (12).

It is therefore likely that the positive effects of anthocyanins, or anthocyanin-rich products, on endothelial function in humans is mediated via the activation of NO-cGMP signaling and reducing the plasma levels of cellular adhesion molecules, thereby lowering the progression towards atherosclerosis.
ANTIOXIDATIVE EFFECTS

Oxidative damage plays a significant role in the progression of CVD [16]. As described above, the presence of NO is needed to maintain normal endothelial function. However, reactive oxygen species can interact with NO and reduce its bioavailability [17]. Anthocyanins are powerful anti-oxidative compounds, which have the ability to scavenge radicals and chelate metal ions preventing the cells from oxidative damage [18].

Blackcurrant juice was assessed for its anti-oxidative potential. Blackcurrant juice supplementation in healthy subjects [250 mL juice per day for 1 week] showed significantly increased serum anti-oxidative status (serum SH groups and PON1 lactonase activity) [19]. A recent human intervention study with bilberry pomace extract demonstrated the anti-oxidative effects at the molecular level. It was found that the metabolite phloroglucinol aldehyde (PGA), formed after digestion of the anthocyanin-rich extract activates the NRF2/ARE stress-response mechanism. The transcription factor nuclear factor E2 (Nrf2) is activated by anthocyanins. It moves to the nucleus and activates the antioxidant response element (ARE) sequence. Upon activation several genes coding for anti-oxidative and anti-inflammatory factors are expressed [20].

INFLAMMATION

Inflammation in CVD plays an important role in the progression of atherosclerosis. During oxidative stress lipoproteins in the blood are oxidized, and can be taken up more rapidly by macrophages in the blood. Oxidized lipoproteins can harm vascular endothelial cells as the loaded macrophages can turn into foam cells [13]. These foam cells attach to the endothelial wall and become fatty streaks. More inflammatory mediators will be attracted to this place, the fatty streak will increase, and will be strengthened with fibrin to form a plaque. The inflammatory response of the body involves macrophages, which play a central role in the regulation of NO, prostaglandins and cytokines. Transcription factor NF-KB plays a central role in the inflammatory response.

Activation of NF-KB leads to activation of target genes, and pro-inflammatory mediators are secreted [21].

Supplementation with bilberry juice in subjects with at least one risk factor for CVD (330 mL juice per day for 4 weeks) resulted in a significant decrease in the plasma concentrations of inflammatory mediators CRP, IL-6, IL-15 and MIG. All of these are target genes activated by NF-KB [21].

Several randomized placebo-controlled trials supplementing purified anthocyanins from bilberry and blackcurrant showed positive effects on inflammatory mediators. Supplementation in healthy subjects [300 mg anthocyanins per day for 3 weeks] resulted in a significant decrease in NF-KB controlled pro-inflammatory mediators. IL-4, IL-13, IL-8, “regulated upon activation, normal T cell expressed and secreted” (RANTES), and IFN-α were significantly decreased after anthocyanin supplementation. No change was found in TNF-α, CRP and IL-1β. IL-4, IL-8 and IL-13 are pro-inflammatory cytokines that induce NF-KB, and IFN-α also induces NF-KB. Within the same study monocytes were exposed to the same purified anthocyanins and a LPS-induced activation of NF-KB was significantly suppressed. This shows that the anthocyanins are most likely to inhibit the NF-KB activation, and decrease the amount of pro-inflammatory mediators [22]. A similar study in subjects with hypercholesterolemia [320 mg anthocyanins per day for 24 weeks] also found anti-inflammatory effects; decreased levels of CRP and IL-1β, cytokines which were not found to be affected in healthy individuals [15, 22]. The exposure and target group could explain this difference. In a HepG2 cell line assay the single anthocyanins C3G and D3G inhibited the IL-6 and IL-1β induced CRP production in a dose dependent manner. The anti-inflammatory effects found with simultaneous exposure of C3G and D3G were stronger compared with the effects of exposure to the single anthocyanins, indicating a synergistic effect [15].

Not all the studies done with purified anthocyanins gave positive effects on inflammatory markers. Another randomized, placebo-controlled, double-blind trial performed in pre-hypertensive subjects [640 mg purified anthocyanins from bilberry and blackcurrant per day for 3 weeks] showed no significant difference in anti-inflammatory markers [23]. The lack of effect can be due to the small sample size, low power of the study, target group and/or time window.

It is most likely that the anti-inflammatory effects of anthocyanins, or anthocyanin-rich products are mediated either via the direct inhibition of NF-KB or via the inhibition of its activators, preventing the activation of pro-inflammatory reactions in the body.

ATHEROSCLEROSIS

Atherosclerosis is characterized by thickened artery walls, narrowed by plaques consisting of white blood cells, dead cells, triglycerides and cholesterol, which impedes the blood flow. The process starts with oXLDL uptake by macrophages. This can be caused by either an increased uptake of LDL cholesterol, or a reduced presence of HDL.
HDL facilitates the reverse cholesterol transport, meaning that it promotes the efflux of cholesterol from macrophages, foam cells and atherosclerotic plaques, so that it can be transported to the liver and excreted as bile (24). This is shown in Figure 3.

Short-term supplementation with blackcurrant juice in healthy subjects [single dose of 250 mL 20% juice] does not show a significant effect on plasma triacylglycerides and non-esterified fatty acid concentration levels (13). A similar study with blackcurrant juice in healthy subjects (250 mL 100% juice for 1 week) showed a significant decrease in macrophage cholesterol content; however the cholesterol efflux rate from the cells was not affected. As the efflux rate has not changed, it is most likely that the uptake of cholesterol has been inhibited (19). The difference between the two results could be due to dose, exposure time and the subjects on which the study was performed.

Supplementation with purified anthocyanins from a mixture of bilberry and blackcurrant in dyslipidemic subjects [320 mg per day for 12 weeks] showed significant increases in HDL cholesterol, decreases in LDL cholesterol, and increased cellular cholesterol efflux to serum. The mass and the activity of plasma cholesterol ester transfer protein (CETP) was significantly decreased and its change was correlated with the change in LDL cholesterol, while the change in HDL cholesterol was found to be correlated to the cellular cholesterol efflux to serum (24). A similar study with the same anthocyanins [320 mg per day for 12 weeks] in hypercholesterolemic subjects showed significant decrease in LDL cholesterol, and an increase in HDL cholesterol. The change in cGMP (marker of endothelial function) and HDL facilitates the reverse cholesterol transport, meaning that it promotes the efflux of cholesterol via macrophages, foam cells and atherosclerotic plaques, so that it can be transported to the liver and excreted as bile (24). This is shown in Figure 3. PLDL stores cholesterol in the blood stream HDL regulates LDL storage and promotes excretion

**Figure 3. Atherosclerotic plaque formation.**

**CONCLUSION**

Anthocyanins have been revealed to be powerful compounds in the support of heart health because of their action on multiple targets. In the pathology of cardiovascular disease they improve endothelial function, anti-oxidative markers, anti-inflammatory effects, and also improve the lipoprotein profile. These targets cannot be seen in isolation, as for instance an improvement found in the lipoprotein profile is also found to be correlated to endothelial function (12). By influencing these targets, anthocyanins contribute to the maintenance and improvement of heart health. Whether the effects observed are directly caused by the anthocyanins themselves, or their metabolites is a current focus of research. The bioavailability is found to be extremely low in humans; <0.1% of the intact anthocyanins are excreted in urine. This indicates that after ingestion anthocyanins undergo an extensive first-pass metabolism, and enter the systemic circulation as metabolites. The concentration of the phenolic acid metabolites is found to be significantly higher in the blood plasma than the concentration of the parent compound. It is therefore likely that not only the parent compounds, but also the metabolites are bioactive, and thus likely to be responsible for the beneficial health effects [25, 26].

Although bilberries and blackcurrants are important natural sources of anthocyanins and other phenolic compounds, they require a relatively long exposure time before a positive effect on heart health is observed. Short term studies gave weak or modest results at best (19), whereas studies performed with purified anthocyanins [320 mg to 640 mg/day, over several weeks] showed positive effects on several aspects of cardiovascular health. This is most likely due to the higher dosages of anthocyanins found in supplements. An edible 100 gram portion of bilberry contains ca. 430 mg and blackcurrant ca. 270 mg total anthocyanins (6). Consumption of an effective anthocyanin dose over the long term only through berries would require a large daily intake, which may stress the digestive system, especially with tannin-rich berries. The use of supplementation with purified anthocyanins, or extracts containing high concentrations of anthocyanins, should be explored in more detail to determine their effects on long term supplementation in large, well-designed placebo-controlled randomized design trials with surrogate markers or clear clinical end points of CVD, while simultaneously monitoring the absorption and metabolism (26-29). The current available studies are encouraging and...
have shown that supplementation using purified anthocyanins can lead to effective dietary levels of anthocyanins, which have been found to have positive effects on biomarkers of human heart health.

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Vitamin K2 for heart health
Important for preventing calcification of heart and blood vessels

KEYWORDS: Vitamin K2, heart health, cardiovascular disease, vascular calcification, calcium.

Abstract
The strong link between the silent arterial calcification and increased risk for cardiovascular events suggests needs for preventive actions. Research points to a pivotal role for the Matrix Gla protein (MGP), one of the strongest natural inhibitors of vascular calcification, in preventing calcification. Vitamin K2 (menaquinone-7, MK-7) is a key factor for MGP activation. However, vitamin K2 deficiencies are quite common in the general population. Newer research suggests that intake of vitamin K2 in dietary supplement doses may increase MGP activation and contribute to prevention of calcification of heart and blood vessels in healthy people.

INTRODUCTION
Cardiovascular disease (CVD) - large challenges to the society
Cardiovascular disease (CVD) is the number one cause of death globally; more people die annually from CVD than from any other cause. CVD is closely related to lifestyle factors. Heart disease and stroke can to a large extent be prevented through a healthy diet, regular physical activity and avoiding tobacco smoke.

An estimated 17.3 million people died from CVD in 2008, representing 30% of all global deaths. Of these were an estimated 7.3 million due to coronary heart disease and 6.2 million due to stroke (1). CVD causes 47% of all deaths in Europe, and is the main cause of death for both women and men in most countries. Overall CVD is estimated to cost the EU economy nearly € 196 billion annually (2). In the US it has been estimated that in the next twenty years the cost of medical care for heart disease will rise from $273 billion (2010) to $818 billion (2030). In addition, heart disease will result in cost related to productivity; $172 billion in 2010 increasing to $276 billion in 2030 (3). Due to the aging US population it is estimated that by 2030, almost 4% of US adults will have had a stroke during their lifetime (4).

Cardiovascular calcification - a risk factor for CVD
CVD is caused by disorders of the heart and blood vessels. CVD includes coronary heart disease (heart attacks), cerebrovascular disease (stroke), raised blood pressure (hypertension), peripheral artery disease, rheumatic heart disease, congenital heart disease and heart failure. Heart attacks and strokes are usually acute events and are mainly caused by a blockage that prevents blood from flowing to the heart or brain. The most common reason for this is a build-up of fatty and calcium rich deposits on and in the inner walls of the blood vessels that supply the heart or brain - known as atherosclerosis. There are two major types of calcium mineralization: calcification associated with atherosclerotic plaque (calcification of the tunica intima), and medial arterial calcification (in vascular smooth muscle cells, VSMC, in the vessel wall). Figure 1 (for a review on calcification of the arteries, see (5)). Arterial calcification was initially referred to as Mönckeberg’s sclerosis and both the medial and internal elastic lamina (layer of elastic tissue layer between the intima and media tunica) are involved (6). Vascular calcification reduces arterial elasticity and result in stiffening of the vessels.

![Calcification of the arteries is closely linked to increased risk of cardiovascular disease (7-11). In patients, an inverse relationship has been shown between serum vitamin K levels and calcification progression.](image-url)
relationship between calcification of vessels and survival is demonstrated [12]. Vascular calcification (VC) is frequent in the general population and is increasing with age. Recent studies points to a key role of Matrix Gla protein (MGP) in prevention of VC [13-15]. MGP is dependent on vitamin K, in particularly vitamin K2 for its function [13-15]. This paper will discuss the role of MGP and vitamin K2 for prevention of VC.

MATRiX GLA-PROTEiN, MGp, A STRONG INHIBIToR OF VESSEL CALCIFICATION

One of the strongest inhibitors of vessel calcification is the vitamin K-dependent protein MGP [16, 17]. The precise mechanism of MGP is not known, but a major role is inhibition of soft-tissue calcification. MGP is expressed in bone, lung, kidney, arteries and cartilage [18, 19]. Just like osteocalcin in bone, MGP is dependent on vitamin K for its function; vitamin K is a co-factor for the enzyme γ-glutamyl-carboxylase which activates “so-called” Gla-proteins by introducing carboxyl-groups in the proteins. In the activated form, the Gla-proteins can bind calcium and contributes to control the calcium distribution in the body. Vitamin K is necessary for the activation to take place and is crucial for the body’s ability to regulate calcium. Activated MGP binds calcium, preventing calcium deposits in arteries (Figure 2).

MGP was first described by Price [20], and its presence in arteries and in atherosclerotic plaques has later been demonstrated [21-23]. Lou and coworkers showed that mice lacking MGP developed to term, but died within two months as a result of arterial calcification which lead to blood-vessel rupture [24]. In accordance with this, Price was able to demonstrate that in warfarin treated rats, inhibiting vitamin K dependent MGP activation, the animals developed valves (21).

MGP is carboxylated in five residues and may also be phosphorylated at serine-residues [19]. The function of the phosphorylation is not exactly known but is believed to be connected to protein secretion. The plasma level of circulating de-phosphorylated, under-carboxylated MGP (dp-ucMGP, inactive) correlates well with vascular mineralization and a low vitamin K status [19]. In accordance with this, activated MGP [in the presence of vitamin K2] prevented reversed calcification of vessels in animal models and human cells in vitro [15, 19, 25-26]. Furthermore, Gla-peptide fragments of MGP inhibited formation of hydroxyapatite crystals in vitro [27]. An inverse relationship between dp-ucMGP (inactive) and survival in cardiovascular patients indicates an important role of activated MGP in preventing vessel calcification [28]. Theuwissen and co-workers summarizes studies showing a close correlation between high circulating levels of dp-ucMGP and calcification [29]. A recent publication supports these findings; in a study in 577 community-dwelling older people with a follow up of 5.6 years, high plasma dp-ucMGP was associated with increased risk of cardiovascular disease independent of classical risk factors and vitamin D status. The effect was attributed to low vitamin K status [30]. The same result were found in hemodialysis patients; a 4.5 fold higher serum levels of dp-ucMGP and a 8.4 fold higher under-carboxylated osteocalcin (ucOC) was found compared to controls [31]. The studies confirm that both in the general population as well as in hemodialysis patients vitamin K deficiency is common and may lead to vascular calcification.

VITAMIN K2- A KEY PLAYER FOR OPTIMAL CARDIOVASCULAR HEALTH

Vitamin K comprises a number of structurally related compounds including phylloquinone (vitamin K1) and menaquinones (vitamin K2s); menaquinone-4 (MK-4) and menaquinone-7 (MK-7) being the most important. Figure 3 shows the molecular structure of all-trans MK-7, the active form of MK-7 [32].

The major differences in effect between vitamin K1, MK-4 and MK-7 are due to differences in absorption, lipid transport in blood and tissue distribution [33-35]. The half-life for MK-7 is substantially longer than for vitamin K1 and MK-4; 72 hours vs. 1-2 hours, resulting in higher blood and tissue levels. As a result, MK-7 has better ability to activate Gla-proteins in extra hepatic tissue [i.e. bone and vessels] even in microgram doses [34].

PREVENTING SOFT TISSUE CALCIFICATION – MAY SUFFICIENT INTAKE OF VITaMIN K2 MAKE A DIFFERENCE TO PUBLIC HEALTH?

Epidemiological studies have shown a relationship between intake of vitamin K2 and positive effects on cardiovascular
health. In a study in the Netherlands [The Rotterdam Study – 2004], involving 4807 Dutch men and women followed for 8-11 years, it was demonstrated that intake of food with high content of vitamin K2 (menaquinones 4-10) dramatically reduced the risk of cardiovascular diseases and mortality (Figure 4) (36). Vitamin K1 had no effects on outcome in this large study. In a follow-up study in 564 postmenopausal women using food-frequency questionnaire to estimate intake of vitamin K, it was demonstrated that high intake of menaquinones, but not vitamin K1 was associated with reduced coronary calcification (37).

A similar study in 16057 women showed that there was an inverse correlation between intake of vitamin K2 (menaquinones MK7, MK-8 and MK-9) and the risk of coronary heart disease (CHD). Vitamin K1 intake was not associated with reduction of CHD (38). These data supports earlier findings that dietary vitamin K1 had little or no effect on coronary calcification (39).

The importance of vitamin K2 for cardiovascular health was recently documented in a double-blind placebo controlled study in 244 healthy postmenopausal women. After daily intake of 180 µg MK-7 for three years the treatment group showed improved arterial stiffness compared to placebo. The improvement was highest in women having high arterial stiffness at start of the study (40).

WHAT HAPPENS IN PEOPLE ON ANTI-COAGULANT TREATMENT (SEVERE VITAMIN K DEFICIENCY)?

Oral anti-coagulants or vitamin K-antagonists like the coumarins (warfarin) are used globally for primary and secondary prevention of both arterial and venous thrombosis (41). Coumarins interfere with the activation of vitamin K-dependent coagulation factors, thereby lowering the ability for blood clotting. While warfarin may prevent stroke and pulmonary embolism, it may possibly contribute to complications associated with low vitamin K activity, such as osteoporosis, bone fractures, and calcification of arteries (42-48).

The coagulation factors are activated in the liver, while MGP and OC are activated in the vessel wall and bone tissue, respectively. As warfarin inhibits the vitamin K-cycle, the inhibition takes place not only in the liver. This implies that warfarin may lead to vitamin K deficiency in peripheral tissue, potentially giving serious side-effects. The side-effects of warfarin treatment on Gla-proteins in extra-hepatic tissue have been reviewed by Danziger (49).

In a cross-sectional study in middle aged long term coumarin users and a matched control group, it was demonstrated that chronic coumarin therapy is associated with enhanced vascular calcification (47). It was suggested that the effects were related to the inhibition of MGP activation, probably due to vitamin K2 deficiency. In a recent study, use of anti-coagulants was associated with negative effects on cardiovascular health (48). The authors suggest that chronic use of vitamin K antagonist may enhance potentially harmful coronary calcification in elderly low-risk atrial fibrillation patients. The possible link between VC and anti-coagulant treatment is discussed by Chatrau and co-workers (50).

VITAMIN K IN FOOD – IS THERE A DEFICIENCY IN THE GENERAL POPULATION?

Vitamin K1 found in greens is considered as the major dietary source of vitamin K, accounting for approximately 90% of total vitamin K intake (50). Vitamin K2, menaquinones, is found in animal products and fermented food products such as cheese. The content of menaquinones in the daily food intake has decreased over the past hundred years. In the previous century it was common to eat fermented products like “sauerkraut”, cured fish and aged cheeses. Today the most efficacious vitamin K, MK-7, is only found in high concentration in the natto dish (fermented soybean) in Japan. However, the content vitamin K2 in Western diet is low and it has been suggested that there is a vitamin K2 deficiency in the population in most countries in Europe and in the US (51).

The intake of K vitamins is sufficient for 100% activation of the clotting factors in the liver in the healthy population (33, 29). In contrast to the coagulation factors, several studies have demonstrated that both OC and MGP in serum are not fully activated in the general population (only 10-40% activated) and that supplementation increases the degree of activation (29, 31, 53-56). Potentially, extra-hepatic carboxylation of Gla-proteins contribute to better health (29). As mentioned, elderly community-dwelling people showing vitamin K-deficiency (high dp-ucMGP) followed for 5.6 years showed increased risk of incidence of CVD (30). Theuwissen and coworkers (29) showed that intake of MK-7 in supplement doses (i.e. 90 µg and above) resulted in significantly increase in carboxylated OC and MGP in circulation.

Very few studies have focused on the vitamin K status in children. In a recent study (57) it was found that children have a low level of dp-ucMGP which increased progressively with age. After supplementation of children (45µg/day for seven weeks, 6-10 years) or adults (90µg/day for seven weeks, 20-40 years) a significant decrease in dp-ucMGP was found both for both groups compared to placebo groups. The authors conclude that children and adults above 40
years showed the largest tissue-specific vitamin K2 deficiency and may benefit from supplementation (57).

THE CALCIUM PARADOX – THE LINK BETWEEN BONE- AND CARDIOVASCULAR HEALTH

As pointed out, vascular calcification is an independent risk factor for CVD and mortality. Interestingly, osteoporosis shows a co-occurrence with CVD and vascular calcification. Several recent publications demonstrate a link between CVD and osteoporosis (53, 60-63). The link is mainly based on epidemiological studies showing that people with arterial calcification also have increased bone loss and vice versa (64-68). The diseases share common risk factors such as old age and inactivity and the relationship has been regarded as general effect of aging. Recent reviews suggest that CVD and osteoporosis share the same pathophysiological mechanisms (67, 68). Why is there a connection between these apparently different diseases? Both processes are complex and regulated by a.o. vitamins, hormones, lipids and signal proteins, but mineralization is a common denominator.

Calcification of soft tissue such as blood vessels is a process resembling the bone formation process and several research groups have outlined a common mechanism (10, 61, 69). The mineral composition of bone (hydroxyapatite) is chemically very similar to that observed in calcified deposits in atherosclerotic arteries (70, 71). A recent meta-analysis conclude that calcium supplementation for prevention of osteoporosis may actually increase the risk of CVD (72), pointing to the importance of optimal calcium distribution.

The skeleton is built up by bone (hydroxyapatite). Build-up cells (osteoblasts) and break-down cells (osteoclasts) regulate the strength of bones. Bone remodeling (or metabolism) is a lifelong process where mature bone tissue is removed and new tissue is formed. These are slow processes and in adults, only 10% is remodeled every year. Osteoporosis occurs when the activity of bone break-down cells (osteoclast) dominate over the activity of the bone building-cells (osteoblast) resulting in loss of calcium and fragile bones. In healthy bones, osteoblasts produce osteocalcin which binds calcium into bone (hydroxyapatite). Osteocalcin is dependent on vitamin K to be active. Thus, not surprisingly, vitamin K deficiency, resulting in low bone building activity is common in osteoporotic patients.

Vascular calcification is an active process, resulting in calcification within the cells of the vessel walls (as discussed above). During this process vascular smooth muscle cells (VSMC) are developing into osteoblast-like cells and start a mineralization process. What is triggering this process? Studies show that vitamin K2 deficiency (MK-7) results in high concentration of inactive MGP, which contributes to VC. (55, 73, 74).
Development of osteoporosis and CV disease are due to complex pathophysiological mechanisms and many factors are involved. However, one common denominator stands out: vitamin K2 deficiency. Vitamin K2 acts as a cofactor for activating osteocalcin associated with building calcium into bone and for activating MGP, inhibiting calcium accumulation in the vessel walls (67, 68, 73); i.e. this vitamin is a pre-requisite for optimal calcium distribution. Vitamin K2 deficiency may be responsible for the so-called “calcium paradox”; the lack of calcium in the bone and its accumulation in the vessel wall (74). More studies are urgently warranted to evaluate the importance of vitamin K2 in calcium regulation.

CONCLUDING REMARK

Vascular calcification of heart and vessels is to a great extent asymptomatic and leads to CVD and mortality. Today, no treatment for cardiovascular calcification exists. Recent studies suggest that VC is an active process where vitamin K2 plays a key role in prevention and reversal of the process. It has been suggested that circulating level of dp-ucMGP (and ucOC) can be used as a biomarker for vitamin K status; a high concentration of dp-ucMGP (low vitamin K status) may indicate a risk of calcification of soft tissue and a risk of development of CVD (74). Recommendation of vitamin K2 (MK-7) supplementation to the general population could contribute to prevent or reduce risk of CV and risk of bone diseases. Several studies are ongoing for further documentation of the beneficial effects of vitamin K2 to prevent VC and will hopefully confirm the importance of vitamin K2 for prevention of VC (75).

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The vitamin hype includes some hazards
The history of vitamin use and abuse

KEYWORDS: Vitamins, vitamin supplements, dietary supplements, nutrition, health.

Abstract

The discovery of vitamins in the last century has helped to eliminate many nutrient deficiency diseases and diet-related disorders that have plagued humankind since the dawn of civilization. This understanding of the interactions between vitamins and human health has launched the fortified food industry that has led to the addition of vitamins to many of the food products we eat on a regular basis such as milk, bread, juice, and the majority of processed snacks and cereals. Today we also see vitamins and related supplements sold in mega-doses, marketed by the media as the universal cures or preventions for nearly every conceivable ailment such as enhancing immunity, increasing lifespan, boosting sexual drive, improving exercise capabilities, or preventing diseases such as cancer or Alzheimer’s disease. This review will briefly summarize the role of vitamin supplements and examine the exaggerated claims and possible dangers of unrestrained vitamin use from a scientific perspective.

The vitamin hype

Throughout the past decades, the use of dietary supplements has increased significantly alongside the growth of the multi-billion dollar dietary supplement industry (1-3). Over half of the U.S. population uses dietary supplements defined as vitamins and minerals and thousands of claimed beneficial natural or synthetic products of diverse origins (3-5). It is clear from these purchasing habits that consumers have placed a considerable amount of value on these dietary supplements, but what is not so clear, and perhaps, what is puzzling, is to try to decipher why and upon what evidence. Why are we buying these products so readily and incorporating them into our every-day routines and health practices and in such massive quantities? What has led us to believe that these concentrated antioxidants, vitamins, natural products, and minerals are the key to reaching optimal health?

Anthropological studies of supplement practices have uncovered that the reasons for use are complex and rooted in the current culture that incentivizes proactively taking responsibility for one’s own health (6-8). While the desire to be a smart consumer committed to improving personal health and prevent illness is impressive, what is troubling is that much of the information about health and science comes from popular press and aggressive marketing strategies, not from legitimate scientific research (9-11). Thus, the way in which dietary supplements are framed by the popular press, advertisements, or other private companies can create societal assumptions about their effectiveness and their safety and ultimately contribute greatly to their consumption patterns (11,12).

The early portrayal of this in effect is with the cover story of the April 6th 1992 issue of Time magazine, entitled “The New Scoop on Vitamins”. The article explained that the traditional medical views of vitamins and minerals were too limited and that vitamins in doses much higher than those usually recommended may protect against a range of illnesses (13). The scientific sources used in the article to support these claims were sparse and included no results from any well-designed longitudinal clinical trials. Yet, the article became the top selling issue of the year and was well received by numerous pharmaceutical companies and lobbying groups such as the National Nutritional Foods Association (NNFA).

The scope of this paper is too narrow to cover all types of dietary supplements such as all dietary antioxidants, nutrients, and micronutrients beyond vitamins. Examples of these dietary agents may include green tea, red wine, flowers, grains, or any dietary antioxidants from plant oils or natural products. It may be that these dietary supplements eventually prove to have health benefits, or at least not add to our risks. However, these dietary agents have not been adequately tested through actual studies to be discussed here in this report.

Instead, the scope of this article will specifically cover the history and science of vitamin supplements and how they have come to play such a major role in the consumer health
industry. Additionally, this article will focus on why, based on existing evidence, there is little justification for the general and widespread use of high dose vitamin supplements.

We need adequate amounts of all thirteen vitamins to survive and to thrive, and the discovery and isolation of vitamins has led to the prevention of vitamin deficiency conditions as well as the eradication of several devastating nutrient deficiency-linked diseases (14-16).

However, the health-conscious American society today has succeeded in eradicating and treating nutrient deficiency associated conditions and diseases to the point where we now live in an excess of vitamin availability. In addition to the vitamins and minerals that occur naturally in the foods we eat, we are also consuming combinations of vitamins and minerals added to ‘enhance’ juices and sports drinks, in fortified or enriched products, and concentrated in supplemental pill forms (17). Nevertheless, there is little to no conclusive long-term research to support that the consumption of vitamins at doses higher than recommended is beneficial to our health in any way, let alone a safe decision.

THE HISTORY AND SCIENCE BEHIND VITAMIN USE

The belief that the diet is a powerful tool for improving health and preventing illness dates back centuries before there was a real knowledge of nutrition. The Ancient Egyptians, for example, ate liver to cure night blindness long before it was known that night blindness was associated with vitamin A deficiency, and sailors on long-distance voyages throughout the 17th and 18th centuries ate citrus foods to avoid scurvy prior to the understanding that the disease was caused by low levels of vitamin C (18).

The term “Vitamine” was officially coined in 1912, by a Polish biochemist named Casimir Funk who was one of many scientists to contribute to the discovery of vitamins throughout the early 19th and mid 20th century (19). The discovery of vitamins helped clinicians recognize that diseases like scurvy, beriberi, rickets, pellagra, and xerophthalmia were caused by specific vitamin deficiencies as opposed to infections or toxins (20, 21). It was through clinical observations of vitamin deficiencies that chemists were able to isolate and characterize the individual vitamin structures.

Vitamins are defined today as essential nutrients that are needed in small amounts for the normal growth and development of organisms (21). There are thirteen universally recognized vitamins that must be obtained through the diet because they cannot be synthesized in sufficient quantities by the body; vitamins include vitamin A, C, D, E, K, B₁₂, B₉, B₆, B₃, B₂, B₁, B₁₁, and B₁₁₁. Vitamin D is an exception since its precursor cholecalciferol, can be synthesized by the skin with adequate sunlight and can be converted in the kidney to vitamin D. Vitamins are further categorized into fat-soluble vitamins (vitamins A, D, E, and K), which are stored in the liver and adipose tissue, and water-soluble vitamins (vitamins B and C), which are readily excreted by the body. For fat soluble vitamins, the vitamin can become sequestered in fatty tissues, or the opposite insofar as deficiencies might take a very long time to appear since the vitamin stores are accessed over a very long time period (21-23).

Up until the mid-1930’s, vitamins were obtained through food intake alone and levels were regulated by changes made to the diet. However, since the mid 20th century, vitamins have been produced as commodity chemicals made widely available as inexpensive semi-synthetic and synthetic sources of multivitamin dietary supplements and as additives in fortified foods (24). This transition rapidly shifted the focus from adequate vitamin intake to high dose or “mega dose” vitamin intake in order to maximize health outcomes. The words “vitamins” and “antioxidants” have become synonymous to “health” and “fitness” and the adage that “the more vitamins we consume, the better” has been made independent from modern scientific understanding and solid clinical data.

Many of the results showing harmful long-term health effects of seemingly safe vitamins or supplements are met with strenuous disbelief. Despite these findings, the dietary supplemental industry continues to grow with the creation and sales of new pills, serums, drinks and powders containing vitamin concentrations way beyond the Recommended Daily Allowances (RDAs) established by the Food and Nutrition Board of the National Academy of Sciences (25-26).

Advocates will say that all of the 65,000 dietary supplements, including vitamin products that are currently on the market, represent a vast amount of new storehouse of health waiting to be tapped by the consumer. Empowered consumers can read up on boastful new claims, but they may also be unproven and anecdotal reports regarding anything from the prevention of cancer with daily multivitamin use, increased fitness performance with antioxidant supplements, increased immune function with vitamin C pills to the latest herb cure for depression.

There is no contest that these vitamins in their elemental or natural form may be associated with positive health effects such as the prevention of diseases, increased fitness ability, and immune function. However, countless studies have shown that vitamins in supplement form may not have identical health effects as vitamins consumed through their natural food sources (27, 28). While not vitamins, glucosinolates, an anticancer compound, provide a valuable example of how beneficial chemicals in foods can be absorbed differently by the body when eaten as real foods in comparison to when they are consumed in store bought supplements. To illustrate, a study in 2011 found that subject given fresh broccoli florets absorbed and metabolized seven times more glucosinolates than the subjects given isolated glucosinolates in capsule form (27).

Additionally, while several studies have shown antioxidants may protect against free radicals involved in oxidative stress associated with many diseases, cognitive impairment, dementia, and byproducts of high intensity exercises (29-31), additional studies have shown the opposite effect to be true with antioxidants in high-dose supplement form. Specifically, high doses of Vitamin C has been shown to diminish muscle growth with strength training (32,33) and antioxidant supplement combinations have been shown to blunt the signaling of molecular pathways and mitochondria creation, necessary for exercise training adaptations (34, 35). Additionally, high doses of vitamin C and calcium have been shown to be associated with an increased risk of cancer (36, 37).
One explanation is that these inconsistencies are most likely due to other chemicals naturally found in food products that may improve nutrient potency and absorption by the body. There is another explanation, specifically in the case of antioxidants discussed above, that has been hypothesized by a number of scientists referred to as the “antioxidant paradox”. It explains that while we associate free radicals with destructive activity like damaging DNA and interfering with cellular membranes, free radicals also have some vital qualities like killing bacteria, eliminating new cancer cells, and acting as messenger initiators for many necessary biochemical processes (38). Thus, as antioxidants are known to absorb and deactivate these free radicals, most likely, when people are taking antioxidants such as vitamin C and E in large concentrations they disrupt the balance between free radicals and antioxidants to the point that critically necessary biochemical processes such as immune function, cell communication, and muscle growth are interrupted (39).

A way to better demonstrate the difference in antioxidant doses naturally found in foods versus in supplemental form is to compare the concentrations of vitamin C. For a reference, the RDA for vitamin C for a non-smoking adult is 60 milligrams per day. The amount of vitamin C in one orange is between 50-75 milligrams and in one cup of orange juice is about 75-90 milligrams. In contrast, a typical vitamin C tablet contains 1000 milligrams accounting for over 1,000% of the RDA, and some labels advise taking two tablets per day (40). Marketers encourage consumers to continue high dose antioxidants by claiming these products contain higher antioxidant scores, better solubility profiles or even stronger or longer biological activity. However, the basis of this logic is flawed because many claims of antioxidant success have based only in vitro studies, which cannot observe long-term side effects or toxicities in vivo.

One example is The Physician’s Health study that followed a total of 14,641 US male physicians above the age of 50 receiving different vitamin supplement combinations from 1997 until 2007 with follow up studies lasting until 2011. Results from this longitudinal study found that there were no immediate or long-term effects on the risk of total cancers with vitamin E or C supplementation. Also, there was no difference in cognitive performance or verbal memory from the individuals that took daily vitamins versus the individuals that took placebos throughout this study (49).

In addition to the null results of vitamin supplement use, several large clinical trials such as the Alpha Tocopherol Beta-Carotene Cancer Prevention Trial, The Beta-Carotene and Retinol Efficacy Trial, The Women’s Health Initiate, and The Iowa Women’s Health Study have found that the regular use of vitamin and mineral supplements including multivitamins, vitamin B6, vitamin E, folic acid, supplemental iron, magnesium, zinc and copper, resulted in increased incidences of mortality and diseases including several types of cancers (48, 50-52).

There have also been numerous studies that show that calcium supplementation with or without vitamin D increases the risk of cardiovascular events, specifically, myocardial infarctions (53-55).

An important thing to consider, that is often overlooked, is that individual factors such as age, gender, weight, total vitamin intake, medication use, predisposition for disease, exercise frequency, and other environmental exposures such as cigarette smoke can greatly affect the way in which each individual tolerates vitamin intake (56). In other words, vitamin use in combination with predisposition for cancer or tobacco use for example have been shown to increase the likelihood for possible harmful health effects (57).

Also, of great interest, is the data from the National Health and Nutrition Examination Survey (NHANES) of 2003-2006 that showed that individuals who use dietary supplements containing vitamins and minerals tend to have higher intakes of fortified foods and vitamin-rich diets from natural food sources than non-vitamin users to begin with (1, 58). Thus, it is those individuals who are already getting adequate vitamin intake who are consuming the extra, unnecessary mega-doses of vitamins in supplement form. This combination can be dangerous as users who reach levels above the recommended Upper Limits (UL) of vitamin intake for nutrients such as folic acid, vitamins A, B6, C, calcium, iron, zinc and magnesium increase the potential for nutrient toxicities and secondary complications such as decreased organ function and increased cancer incidence (2, 15, 17, 59).

The Dietary Supplement Health and Education Act (DSHEA), signed in 1994, classified dietary supplements as a subcategory of food. There is a history of controversy over this legislation, specifically, because it relies solely on the dietary supplement manufacturers to insure the quality and safety of their products. Vitamin supplements require no standardization or testing for safety to be submitted to the US FDA before they are made available on the market (57).

The leniency of the DSHEA also allows labels on vitamin bottles to include claims about structure and function of the vitamins as long as they do not claim to treat or cure certain diseases (57). This leads to confusion for consumers who are trying to decipher between
marketing claims and scientific descriptions on labels; many consumers have been misled to believe that dietary supplements including vitamins can be substituted for physician prescribed medications (60).

Lastly, unlike in most European countries where the daily dosage of a vitamin supplements cannot exceed 300% of the recommended daily allowance as regulated by the government, marketers can dictate dosage and use, bypassing any dosage regulations that are normally in place for determining the use of drugs in the United States (61).

There have been attempts to regulate the dietary supplement industry more closely, such as the proposition of The Dietary Supplement Safety Act of 2010 by Senators John McCain and Byron Dorgan that would have required dietary supplement manufacturers to register all products with the US Food and Drug Administration (FDA) and fully disclose all product ingredients upon registration (62). The Dietary Supplement Safety Act never became law.

CONCLUSION

High dose vitamin supplements are challenging to study because of different study conditions, durations, doses and agents used, diseases measured, and dietary histories of subjects involved in the research conducted thus far. One can easily search the literature and locate studies showing both positive, negative and null findings. While those contradictions continue to exist (as common with many emerging research topics), what many experts can agree on is the best way to get our vitamins is from the real foods that are naturally rich in vitamins, minimally processed, and contain countless beneficial chemicals beyond vitamins. This method for primary vitamin intake insures a built-in system of “checks and balances” because while there is variability in nutrient intakes and needs across the population, it is difficult to reach vitamin toxicity levels from naturally vitamin dense foods alone. In conclusion, the hype for vitamins may still remain, but the hype should shift from reaching into bottles for capsules of vitamins to reaching across the population, it is difficult to reach vitamin toxicity intake insures a built-in system of “checks and balances”.

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amino acids absorbed from the gut in a certain chemical form. However, during the processing of food, some amino acids such as lysine and alanine are susceptible to chemical modification. Lysine is especially important, because lysine is one of nine essential amino acids that humans cannot synthesize themselves. A fixed pattern of these essential amino acids is required to maximize protein synthesis in tissues, such as muscle and bones. In a grain-rich diet, lysine is often the first-limiting amino acid (3).

Lysine has a side chain amino group which can easily react with reducing sugars such as fructose, lactose and glucose. During processing (especially heat treatment) and during storage, Maillard reactions occur (5). A Maillard reaction is a chemical reaction between reducing sugars and amino acids. The Maillard reaction can be divided into three different stages: initial, intermediate and late. A late or advanced Maillard reaction is recognized in browning when baking products. The early Maillard reaction results in the formation of Amadori compounds in which lysine is no longer nutritionally available (6). Loss of available lysine is the most significant consequence of the Maillard reaction and it is of great importance in those foods where lysine is the limiting amino acid (7).

How to preserve amino acids availability
An easy strategy to prevent early Maillard reactions is to choose a protein source with low or no reducing sugars. This means no sugar is naturally present (such as lactose in different protein sources) or reducing sugars are added to the product. Also check table 1 for the protein and lactose content of different protein sources.

Protein is not just protein. The quality of a protein source can be determined through three characteristics: the amount of protein in food, the amount of indispensable amino acids in the protein and digestibility. Dietary protein quality can be assessed using protein quality parameters. The FAO/WHO recommends PDCAAS or Protein Digestibility Corrected Amino Acid Score as a suitable method for determining dietary protein quality (Figure 1). This score is calculated from the first-limiting amino acid in digestible dietary protein relative to the amino acid requirement in humans (3).

Damage of protein after processing and storage
The available amino acids that can be used for protein synthesis (2) are dietary amino acids. How to retain or prevent the degradation of the amino acids absorbed from the gut is possible through processing and storage.
The body cannot use blocked lysine for protein synthesis

It is assumed that blocked lysine is not biologically available because the covalent bond between lysine and sugar cannot be split by digestive enzymes [8]. Although blocked lysine can be partly absorbed from the digestive tract, it has no nutritional value and will be excreted with urine [5]. Hence, the vast majority of blocked lysine (> 70%) enters the colon where it is predominantly utilized by microorganisms and also affects microbiota composition. This means that blocked lysine will not be used for body protein synthesis in order to maintain or develop structural proteins in muscle and bone [8, 9].

Conclusion

It is increasingly acknowledged that not only amino acid composition, but also the availability of essential amino acids, contributes to the quality of dietary proteins. Lysine damage impacts the availability of essential amino acids, especially lysine, for protein synthesis in tissues such as muscle.

The older population is decreasing dietary protein intake, so for this population it is of extreme importance that all the protein that are ingested is available for the body. Milk protein is known as a high quality protein source. Also proteins extracted from milk, such as caseinate are suitable as a protein enrichment in the diet of elderly. Caseinate has several benefits: it has a low lactose content, it has all amino acids available and the quality remains after processing.

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[www.beneo.com](http://www.beneo.com)

### CHS Inc. Soy & Canola Products Improving Food & Health

CHS Inc. offers Soybean and Canola Oil, Defatted Soy Flour, Soy Protein Isolate, Textured Soy Flour and Analogs, and Soy Protein Isolate. We offer both GMO and Non-GMO options and have received Non-GMO Project Verification status as well as Cert ID EU Certification on many of our soy products.

[www.chsinc.com/foods](http://www.chsinc.com/foods)

### DKSH - Innovative food and beverage ingredients

DKSH is a leading food and beverage ingredients distributor and global provider of Market Expansion Services for the beverage, dairy, confectionery and bakery, nutritional supplements, processed food and food service industries.

[www.dksh.com/fbi](http://www.dksh.com/fbi)

### DSM brings digestive health solutions to FIE 2015

At this year’s FIE, DSM brings its range of digestive solutions, highlighting nutritional and functional ingredients and enzymes backed by science to support digestive health. This allows products to be tailored to popular consumer health requirements. Visit DSM’s stand to find out its latest solutions for digestive health, sugar reduction and more.

[www.dsm.com/food](http://www.dsm.com/food)
Meet the world’s leading ingredients suppliers in Paris at FIE

| Booth 6G110 | Evonik - a premier supplier of advanced food ingredients, processing aids and functional coatings  
From the anti-caking agent in paprika to natural flavor extracts, amino acids, sodium reduction and functional coatings for food supplements — Evonik is widely represented in the food industry. Our unique ingredients bring value into nutritional products and in the process promote health and well-being. Potential applications range from infant formulas to sports nutrition and dietary supplements.  
www.evonik.com/food-ingredients |
|---|---|
| Booth 6K19 | FrieslandCampina Domo introduces Vivinal GOS powder at Fi Europe  
Vivinal GOS Powder is a new product for infant nutrition applications. This galacto-oligosaccharide high purity powder contains high levels of galacto-oligosaccharides and low levels of mono-saccharides. This product gives more flexibility in recipe formulation, provides higher process flexibility and reduction in operational costs.  
www.vivinalgos.com |
| Booth 6K12 | Team up with the expert  
Collagen Proteins are bridging the gap between different Food categories. GELITA, the expert in all areas of collagen protein applications can merge solutions and create synergies between  
www.gelita.com |
| Booth 6M74 | Protein Fortified: Let us show you how  
We deliver the promise of dairy – whey protein, lactose and milk powder. From bars to beverages, our products provide flexible fortification and nutrition. Hilmar Ingredients is SQF certified and all facilities meet requirements of Global Food Safety (GFSI). We manage our business to balance economic, social and environmental sustainability.  
www.hilmaringredients.com |
| Booth 6F46 | HOCHDORF – for healthy nutrition  
The HOCHDORF Group is a leading Swiss company in development and production of healthy ingredients manufactured from milk, whey, cereals and oil seeds. HOCHDORF are focused on customized casein and whey proteins, dairy fat specialties, base powders, wheat germ specialties (e.g. as a nut replacement) and organic vegetable oils.  
www.hochdorf.com |
| Booth 6F114 | Indena at Food Ingredients Europe 2015  
Indena is further expanding its presence in the food sector with a new and dedicated food division. Oplodex™, a new technology platform for food preservation, will be launched this year at Food Ingredients Europe. Based on innovative combinations of natural polyphenols endowed with strong antioxidant and chelating properties, Oplodex™ range of food ingredients aims to meet industry’s needs and consumers’ requests.  
www.indena.com |
Meet the world’s leading ingredients suppliers in Paris at FIE

Booth 6K76

Innophos-Ingredients for Life™
Innophos, Inc. is a global manufacturer of specialty phosphates with applications in food, beverage, and dietary supplement end markets. Innophos will be featuring solutions for sodium reduction.
Innophos Nutrition offers bioactive mineral and premium botanical ingredients to the nutritional and natural products industries.
www.innophos.com

Booth 6L45

Jungbunzlauer at Fi Europe 2015
Jungbunzlauer showcases innovative solutions to the global food trends sodium reduction and mineral fortification. A further highlight will be sugar reduction: The recent approval of the European Commission of erythritol for beverages opens new opportunities for Jungbunzlauer’s ERYLITE® in the field of energy reduced and “with no added sugars” flavoured drinks.
www.jungbunzlauer.com

Booth 7E18

LECICO will present a range of sunflower lecithin at the FIE Europe
LECICO is a highly specialized company with 30 years of experience and a 100% focus in the lecithin market.
At the FIE Paris the lecithin expert focuses on the increasingly important niche of sunflower lecithin – available in fluid, de-oiled or organic quality.
Also special lecithin products from rapeseed, soy and milk will be showcased.
www.lecico.de

Booth 6M49

Lesaffre Human Care to introduce their newly organic-certified nutritional yeast at FIE 2015
LHC will launch at the upcoming FIE, Lynside® NUTRI ORGANIC. In fact, the company recently obtained an organic certification from Ecocert for this traditional superfood. These nutritional yeasts are an excellent source of protein, B vitamins, fibers and minerals, making it a great nutritional partner for athletes, the elderly, expectant mothers as well as everyone who aspire to lead a healthy lifestyle.
www.lesaffrehumancare.com

Booth 6G19

At FIE 2015 Nexira will focus on Fibregum™, an all-natural & GMO-free source of prebiotic soluble dietary fiber
Fibregum™ offers a guaranteed minimum of 90% soluble fiber with proven prebiotic benefits, exceptional digestive tolerance and low caloric value.
New results demonstrate the beneficial and innovative health benefits of Fibregum™ on the intestinal barrier. The Fibregum™ range is organic certified and verified by Non-GMO project.
www.nexira.com

Booth 6Q55

Omya - Functional Minerals and beyond...
With over 130 years of expertise, Omya provides a portfolio of pure natural minerals according to the most stringent regulatory and quality standards. These are required by the food, pharmaceuticals and cosmetics industries in particular. Omya’s minerals portfolio covers a wide range of applications with established functions.
www.omya.com/fpc
Meet the world’s leading ingredients suppliers in Paris at FIE

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<thead>
<tr>
<th>Booth 6B36</th>
<th>Plantex, first time at FIE</th>
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<tr>
<td>Plantex develops, manufactures and supplies natural ingredients for the Food industry. Specialist in liquid and dry botanical extracts in conventional and Organic Quality, Plantex is your tailor made solution provider for your food applications. Expert of vanilla Bourbon extracts Plantex will present for the first time at FIE 2015 its new FOOD SIGNATURE range of coffee and cocoa extracts.</td>
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<td><a href="http://www.plantex.fr">www.plantex.fr</a></td>
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<tr>
<th>Booth 6D25</th>
<th>Futurals, the next generation of coloring foodstuff</th>
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<tr>
<td>ROHA, the leading manufacturer of colors, is showcasing at FIE Paris the Futurals brand of coloring foodstuffs with the attributes demanded by the market: natural origin and E-number free:</td>
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<tr>
<td>• Futurals PAPRIKA: labeled as “Paprika Extract”</td>
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<tr>
<td>• Futurals BLACK: black coloring</td>
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<tr>
<td>• Futurals RED RANGE: bright red shades for coating</td>
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<tr>
<td><a href="http://www.roha.com">www.roha.com</a></td>
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<tr>
<th>Booth 6K29</th>
<th>Rousselot presents Gelatin and Collagen Peptides Solutions at FIE 2015</th>
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<tr>
<td>Explore the unique and multifunctional properties of gelatin, uncover tailor-made protein solutions and a revolutionary nutrient / food supplement delivery concept with Rousselot. Stop by stand 6K29 to gain expert insights from the global leader in gelatin and collagen peptides.</td>
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<td><a href="http://www.rousselot.com">www.rousselot.com</a></td>
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<tr>
<th>Booth 6P24</th>
<th>Innovative concepts for appealing products</th>
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<td>According to its motto, “We bring life to products,” the Sensient team invites visitors to experience a whole new world of natural flavors and extracts, as well as a broad portfolio of sauces, inclusions, fillings and on-trend value added flavor systems. The company’s portfolio will cover versatile solutions for sweet and savory applications as well as beverages.</td>
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<tr>
<td><a href="http://www.sensientflavors.com">www.sensientflavors.com</a></td>
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<th>Booth 6L111</th>
<th>Groupe SOLACTIS solution: how to match with the hot trends</th>
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<td>Groupe SOLACTIS: a rapidly growing bioscience-based ingredients supplier, presents at FIE new breakthroughs in SOLACTIS® range: COMBO, a unique blend of milk derived oligosaccharides; new health benefits of its “pilar” GALACTOFRUCTOSE and ACTIVATOR for symbiotic compositions</td>
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<tr>
<td><a href="http://www.solactisgroup.com">www.solactisgroup.com</a></td>
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<th>Booth 7B2</th>
<th>Fat free ingredient helps to lower cholesterol</th>
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<td>Vitae Naturals is the main manufacturer of phytosterols esters and the first European producer of certified non-GMO IP naturally sourced Vitamin E. Vitasterol® S-80 WDP is mixed phytosterols microencapsulated especially developed for dairy products. This functional ingredient helps to lower blood cholesterol levels. It is a fat free ingredient.</td>
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<tr>
<td><a href="http://www.vitaenaturals.com">www.vitaenaturals.com</a></td>
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Collagen peptide supplementation in combination with resistance training improves body composition and increases muscle strength in elderly sarcopenic men: a randomised controlled trial

Protein supplementation in combination with resistance training may increase muscle mass and muscle strength in elderly subjects. The objective of this study was to assess the influence of post-exercise protein supplementation with collagen peptides v. placebo on muscle mass and muscle function following resistance training in elderly subjects with sarcopenia. A total of fifty-three male subjects (72.2 (sd 4.68) years) with sarcopenia (class I or II) completed this randomised double-blind placebo-controlled study. All the participants underwent a 12-week guided resistance training programme (three sessions per week) and were supplemented with either collagen peptides (Treatment group [TG]) (15 g/d) or silica as placebo (placebo group [PG]). Fat-free mass (FFM), fat mass (FM) and bone mass (BM) were measured before and after the intervention using dual-energy X-ray absorptiometry. Isokinetic quadriceps strength (IQS) of the right leg was determined and sensory motor control (SMC) was investigated by a standardised one-leg stabilisation test. Following the training programme, all the subjects showed significantly higher (P<0.01) levels for FFM, BM, IQS and SMC with significantly lower (P<0.01) levels for FM. The effect was significantly more pronounced in subjects receiving collagen peptides: FFM (TG +4.2 (sd 2.31) kg/PG +2.9 (sd 1.84) kg; P<0.05); IQS (TG +16.5 (sd 12.9) Nm/PG +7.3 (sd 13.2) Nm; P<0.05); and FM (TG –5.4 (sd 3.17) kg/PG –3.5 (sd 2.16) kg; P<0.05). Our data demonstrate that compared with placebo, collagen peptide supplementation in combination with resistance training further improved body composition by increasing FFM, muscle strength and the loss in FM.

Daniel Königa et al., British Journal of Nutrition, DOI: http://dx.doi.org/10.1017/S0007114515002810

Nano in food and agriculture: regulations require collaboration to ensure safety

An overview of regulatory solutions worldwide on the use of nanotechnology in food and feed production shows a differing approach: only the EU and Switzerland have nano-specific provisions incorporated in existing legislation, whereas other countries count on non-legally binding guidance and standards for industry. Collaboration among countries across the globe is required to share information and ensure protection for people and the environment, according to a JRC co-authored paper. The paper Regulatory aspects of nanotechnology in the agri/feed/food sector in EU and non-EU countries reviews how potential risks or the safety of nanotechnology are managed in different countries around the world and recognises that this may have implication on the international market of nano-enabled agricultural and food products. Nanotechnology offers substantial prospects for the development of innovative products and applications in many industrial sectors, including agricultural production, animal feed and treatment, food processing and food contact materials. While some applications are already marketed, many other nano-enabled products are currently under research and development, and may enter the market in the near future. Expected benefits of such products include increased efficacy of agrochemicals through nano-encapsulation, enhanced bioavailability of nutrients or more secure packaging material through microbial nanoparticles. As with any other regulated product, applicants applying for market approval have to demonstrate the safe use of such new products without posing undue safety risks to the consumer and the environment. Some countries have been more active than others in examining the appropriateness of their regulatory frameworks for dealing with the safety of nanotechnologies. As a consequence, different approaches have been adopted in regulating nano-based products in the agri/feed/food sector. The analysis shows that the EU along with Switzerland are the only ones which have introduced binding nanomaterial definitions and/or specific provisions for some nanotechnology applications. An example would be the EU labelling requirements for food ingredients in the form of ‘engineered nanomaterials’. Other regions in the world regulate nanomaterials more implicitly mainly by building on non-legally binding guidance and standards for industry. The overview of existing legislation and guidances published as an open access article in the Journal Regulatory Toxicology and Pharmacology is based on information gathered by the JRC, RIKILT-Wageningen and the European Food Safety Agency (EFSA) through literature research and a dedicated survey.

European Commission Joint Research Centre
Scientists have developed rice with high folate stability

Researchers from Ghent University succeeded in stabilizing folates in biofortified rice, which can offer a solution to serious health problems caused by folate deficiency in developing countries.

Essential nutrient

The human body is unable to make vitamin B9, better known as folate. Adults need approximately 400 microgram of folates per day to remain healthy, a number which is increased to 600 microgram for pregnant women. Folates are abundant in green leafy vegetables (follum is Latin for leaf), such as spinach and legumes (e.g., beans). Most staple crops, such as rice and other cereals, contain very low amounts of this vitamin.

Inadequate folate intake can have severe effects on human health. In addition to certain forms of anemia, folate deficiency in pregnant women can result in an impaired development of the neural tube (the precursor of the spinal cord) of the embryo. These developmental problems often result in spina bifida: the so-called “cleft spine”. Folate deficiency is also associated with Alzheimer disease, cardio-vascular diseases and the development of a range of cancers. Due to the marginal levels of folate in rice, consumed by about half the world population as sole energy source, folate deficiency is highly prevalent in developing countries. Several studies show that in certain regions of e.g., China and India the occurrence of neural tube defects is at least 10-fold higher than in Western countries.

Unstable molecules

Vitamins are unstable molecules, that degrade easily upon contact with oxygen, light, humidity, increased temperatures and changes in acidity. For this reason, it is important to consume food products, such as vegetables and fruit, as fresh as possible. A lot of vitamins get lost, not only during food processing and preparation, but also during storage. Evidently, these problems occur in harvest products that are stored for a longer period, such as rice grains. These stability problems become more severe in developing countries where the storage in high temperature and high humidity is inevitable.

In 2007, a research team from Ghent University (Belgium), coordinated by prof. Dominique Van Der Straeten, reported the development of a first generation of rice lines with 100-fold higher folate levels as compared to normal rice. This result was achieved through metabolic engineering, the modulation of the biosynthesis pathway of a plant compound. Their new study shows that about half of the folate content in these rice lines degrades after half a year.

Assemblage lines

To tackle this problem, researchers from this lab developed a new rice prototype, in which the folate content remains stable upon long term storage. Again, metabolic engineering was applied. Folate is produced in a plant cell by specific enzymes (molecular machines) that add consecutive changes to a certain start product until a folate molecule is formed. This process is comparable to a car assembly line. By stimulating the production of two enzymes in the folate biosynthesis, researchers created the first generation of rice lines containing high folate levels.

Two strategies

Now, they were able to stabilize this high folate content in a new rice prototype. They applied two strategies. A first strategy comprised the binding of folates with a folate binding protein. This protein is unknown in plants, but well studied in mammals. It occurs in e.g., milk and protects folate from degradation. This is also the way intact folates are passed on from the mother to her infant, to support its development. By expressing a synthetic gene, based on a folate binding protein from bovine milk, in the rice grain, the same principle is applied and folate content remains stable upon long term storage.

A second strategy consisted of the stimulation of the last step in folate production. This step extends the tail of the folate molecule. This promotes cellular retention and binding to folate dependent proteins. Besides enhancing folate stability, the new gene combinations also resulted in folate levels that are up to 150 fold those found in normal rice. Since all genes which were used in this study, were placed next to each other on a single piece of DNA, this piece of generic material can easily be transferred to edible rice varieties. Moreover, it is fairly easy to make combinations with other interesting traits, such as the enhancement of other vitamins or certain minerals, such as iron. This technology can also be used in other crops, both cereals (e.g. wheat, sorghum) and non-cereals (e.g. potato, banana).

Collaboration

This investigation is the result of a close collaboration between the labs of prof. Dominique Van Der Straeten (development and characterization of the new rice prototypes), prof. Willy Lambert and prof. Christophe Stove (development and characterization of the new rice prototypes), dr. Hans De Steur and prof. Xavier Gellynck (study of the socio-economic impact of folate rice). These results have been published in the renowned journal Nature Biotechnology (Blancquaert et al., 2015).

Pioneers

The stability issue is often underestimated or even neglected in biofortification programs. It is obvious that not only high, but also stable vitamin levels are important to tackle vitamin deficiencies. Not only does this study describe the effect of long term storage on the folate levels in the first rice prototypes, it also provides an elegant solution to the stability problem. This solution can be applied, in a customized form, to other crops and vitamins and opens the door for awareness and consideration of vitamin stability in future biofortification studies.

Ghent University
Cocoa flavanols lower blood pressure and increase blood vessel function in healthy people

New studies by the EU-funded FLAVIOLA research consortium show that cocoa flavanols could help maintain cardiovascular health as we age.

Two recently published studies in the journals Age and the British Journal of Nutrition (BJN) demonstrate that consuming cocoa flavanols improves cardiovascular function and lessens the burden on the heart that comes with the ageing and stiffening of arteries. The studies also provide novel data to indicate that intake of cocoa flavanols reduces the risk of developing cardiovascular disease (CVD).

As we age, our blood vessels become less flexible and less able to expand to let blood flow and circulate normally, and the risk of hypertension also increases. Arterial stiffness and blood vessel dysfunction are linked with cardiovascular disease – the number one cause of deaths worldwide. “With the world population getting older, the incidence of cardiovascular disease, heart attacks and stroke will only increase,” says Professor Malte Kelm, Professor of Cardiology, Pulmonary Diseases and Vascular Medicine at University Hospital Düsseldorf and Scientific Director of FLAVIOLA. “It is therefore pivotal that we understand the positive impact diet can have on cardiovascular disease risk. As part of this, we want to know what role flavanol-containing foods could play in maintaining the health of the heart and blood vessels.”

Cocoa flavanols are plant-derived bioactives from the cacao bean. Dietary intake of flavanols has been shown to have a beneficial effect on cardiovascular health but the compounds are often destroyed during normal food processing. Earlier studies have demonstrated that cocoa flavanolate intake improves the elasticity of blood vessels and lowers blood pressure – but, for the most part, these investigations have focused on high-risk individuals like smokers and people that have already been diagnosed with conditions like hypertension and coronary heart disease. These two studies in Age and BJN are the first to look at the different effects dietary cocoa flavanols can have on the blood vessels of healthy, low-risk individuals with no signs or symptoms of cardiovascular disease.

Cocoa flavanols increase blood vessel flexibility and lower blood pressure

In the study published in Age, two groups of 22 young (<35 years of age) and 20 older (50-80 years of age) healthy men consumed either a flavanol-containing drink, or a flavanol-free control drink, twice a day for two weeks. The researchers then measured the effect of flavanols on hallmarks of cardiovascular aging, such as arterial stiffness (as measured by pulse wave velocity), blood pressure and flow-mediated vasodilation (the extent to which blood vessels dilate in response to nitric oxide). They found that vasodilation was significantly improved in both age groups that consumed flavanols over the course of the study (by 33% in the younger age group and 32% in the older age group over the control intervention). In the older age group, a statistically and clinically significant decrease in systolic blood pressure of 4 mmHg over control was also seen.

Improving cardiovascular health and lowering the risk of CVD

In the second study, published in BJN, the researchers extended their investigations to a larger group (100) of healthy middle-aged men and women (35-60 years) with low risk of CVD. The participants were randomly and blindly assigned into groups that consumed either a flavanol-containing drink or a flavanol-free control drink, twice a day for four weeks. The researchers also measured cholesterol levels in the study groups, in addition to vasodilation, arterial stiffness and blood pressure. “We found that intake of flavanols significantly improves several of the hallmarks of cardiovascular health,” says Professor Kelm. In particular, the researchers found that consuming flavanols for four weeks significantly increased flow-mediated vasodilation by 21%. Increased flow-mediated vasodilation is a sign of improved endothelial function and has been shown by some studies to be associated with decreased risk of developing CVD. In addition, taking flavanols decreased blood pressure (systolic by 4.4 mmHg, diastolic by 3.9 mmHg), and improved the blood cholesterol profile by decreasing total cholesterol (by 0.2 mmol/L), decreasing LDL cholesterol (by 0.17 mmol/L), and increasing HDL cholesterol (by 0.1 mmol/L). The researchers also calculated the Framingham Risk Score - a widely used model to estimate the 10-year cardiovascular risk of an individual - and found that flavanol intake reduced the risk of CVD. “Our results indicate that dietary flavanol intake reduces the 10-year risk of being diagnosed with CVD by 22% and the 10-year risk of suffering a heart attack by 31%,” says Professor Kelm.

The combined results of these studies demonstrate that flavanols are effective at mitigating age-related changes in blood vessels, and could thereby reduce the risk of CVD in healthy individuals. The application of 10-year Framingham Risk Scores should be interpreted with caution as the duration of the BJN study was weeks not years and the number of participants was around 100, not reaching the scale of the Framingham studies. That being said, Professor Kelm comments that “the reduction seen in risk scores suggests that flavanols may have primary preventive potential for CVD.” Other longer-term studies, such as the 5-year COcoca Supplement and Multivitamin Outcomes Study (COSMOS) of 18,000 men and women, are now underway to investigate the health potential of flavanols on a much larger scale.

University Hospital Düsseldorf
Evaluation of nutraceuticals effect from in vitro assays

KEYWORDS: in vitro models, human cell lines, nutraceuticals.

Abstract The healthy effects of numerous bioactive natural compounds and the possibility to take advantage of their beneficial physiological functions are highly valued. Although the efficacy of a number of nutraceutical products has already been demonstrated in humans, the properties of many compounds are still unknown and, importantly, there is little information on the properties and potential of their combined use. For these reasons in vitro assays are necessary to identify the mechanisms underlying the protective role of a nutraceutical ingredient alone or in combination with other bioactive molecules. Here we describe examples of in vitro models and methodological approaches used to characterize and evaluate a number of effects of nutraceuticals in living cells.

INTRODUCTION

Studies aimed to identify the potential beneficial use of nutraceuticals have been growing in the last few years. Nutraceuticals are of considerable interest due to their potential nutritional effects as dietary supplements (1-2) and their production should be accompanied by appropriate quality assessment measures (3). Studies are thus needed to assess both their quality and their biological effects when used alone or in combination. For in vitro screening, a crucial step is the choice of the appropriate model that best represents the cellular target of the relevant compound. Human cell lines, with organ-specific properties represent such important experimental systems. Macrophages for acute/chronic inflammation and pain; chondrocyte for articular pain, uroepithelial cells for urinary tract infection, and many others. In vitro models and assays are used to define concentration/doses of compounds and to evaluate the cellular pathways involved in their action.

CYTOTOXICAL SCREENING

The first step to establish a cellular assay is the evaluation of the potential cytotoxic activity of compounds on the selected cell line. Different human cell lines were screened for the potential cytotoxic effect of nutraceuticals through the measure of cell viability and proliferation. These tests are used to define the concentrations of the compound that do not affect cell viability. These assays focus on the evaluation of one of the following features: membrane integrity, cellular metabolite content, mitochondrial and lysosomal functions and cell apoptosis (4). Here we described the MTT assay that measures cell viability and cell proliferation rate (5). In our experiment, we performed a MTT test on human monocytes (THP-1 cells) treated with a nutraceutical (6) at different concentrations (10-50-75-100-500 µM) for 16 hours. In Figure 1A, the results are shown. A strong reduction in viability is seen in cells treated with the higher dose (500 µM) of the compound, as indicated by the lower value of the optical density (OD). In cells treated with the lower concentrations (from 10 to 100 µM) the OD is comparable to control and vehicle.

ANTI-INFLAMMATORY ACTIVITY

Inflammatory response is characterized by the release of pro-inflammatory cytokines and other mediators, as free radicals, and prostaglandin E2 (PGE2), which are synthesized by inducible nitric oxide synthase (iNOS) and cyclo-oxygenase (COX-2). Anti-inflammatory agents can act by modulating the production of cytokines and all the other mediators (7). The anti-inflammatory properties of several natural compounds has long been known and, some of them as curcumin, flavonoids, saponins are generally recommended in the diet of patients suffering from inflammatory diseases such as fevers, pain and arthritis (8-9). Inflammatory conditions (i.e. arthritis and chronic pain) can be mimicked in vitro models by stimulating cells with bacterial lipopolysaccharide (LPS) or IL-1β-induced inflammation. Upon exposure to LPS or IL-1β, macrophages and chondrocytes, release a number of immunoregulatory molecules including tumor necrosis factor-α (TNF-α), IL-6, arachidonic acid metabolites and Nuclear-Factor-Kappa-B (NF-κB) is translocated into the nucleus where it regulates the expression of inflammatory factors.
genes. ELISA test and NF-κB Translocation-Assay are used to measure these inflammatory responses in cultured cells. We next describe how we applied these two assays to study the anti-inflammatory properties of nutraceuticals in monocyte-like cells (THP-1) and Normal-Human-Articular-Chondrocytes (NHAC-kn) upon stimulation with LPS and IL-1β.

**ELISA test**
The Enzyme-Linked-Immunosorbent-Assay (ELISA) has been designed for the in vitro quantification of a range of cytokines. In the experiment illustrated in figure 1B, THP-1 cells, treated with the bioactive substance (Compound-A) for 16 hours, in basal condition or after stimulation with 1 mg/ml LPS for 2 hours, were centrifuged and the cell-free supernatants were collected. Sandwich ELISA was performed on the cell supernatant to determine IL-1β concentration, in accordance with the manufacturer’s instructions (Life-Technologies). ELISA test in cells stimulated or not with LPS, were performed to compare the effect of the compound with the basal concentration of the cytokine. In figure 1B the results obtained in cells without LPS stimulation are shown [left]; these cells present a low basal level of IL-1β (around 5-10 pg/ml). LPS stimulation induced an increase of the concentrations of the IL-1β in all experimental conditions [right]. This increase is reduced upon treatment with the bioactive Compound-A and the effect of the nutraceutical on IL-1β production is more pronounced than the effect anti-inflammatory drugs (Nimesulide and Ibuprofen).

**NF-κB translocation assay**
NF-κB is generally thought to be constitutively active and located in the cytoplasm in most cell types, until induced by a stimulus to migrate to the nucleus. Translocation of NF-κB is a critical step in the coupling of extracellular stimuli to the transcriptional activation of specific target genes. NF-κB is activated by pro-inflammatory cytokines (IL-1α, IL-1β, TNF-α); bacterial toxins (LPS, exotoxin B); viral products (HIV-1, HTLV-1, EBV); and cell death stimuli (O2-free radicals, UV light, γ-radiation) (10-11). Upon cell stimulation, the nuclear localization signal on NF-κB becomes exposed and the protein translocate to the nucleus, where it turns on transcription factors and induces specific gene expression. In cells, the redistribution of NF-κB from the cytoplasm to the nucleus following cytokine stimulation can be easily monitored with fluorescence microscopy. In our experiment we used Normal-Human-Articular-Chondrocytes (NHAC-kn) stimulated with 10 μg/ml IL-1β for 16 hours and treated with increasing doses of Compound-B (12) (125-250-500 μM) for 16 hours and anti-inflammatory drugs (Celecoxib and Diclofenac) as positive controls for 16 hours. As negative controls we used, NHAC-kn cells with and without stimulation treated with vehicle. Cells were then fixed and labeled with NF-κB-p65 antibodies and secondary antibodies conjugated with AlexaFluor488, DAPI was used as a nuclear stain (Figure 2A). NF-κB translocation is calculated by measuring the “ratio” of the average nuclear intensity to average cytoplasmic intensity (Nuc/Cyt Ratio). We demonstrated that cells treated with 500 μM Compound-B have less nuclear NF-κB (reduction of inflammation) than cells treated with vehicle and with an anti-inflammatory drugs (Figure 2B).

**ANTI-OXIDANT ACTIVITY**
Reactive oxygen species (ROS) include free radicals and other molecular species. They are generated during incomplete metabolic reduction of oxygen to water. In physiological conditions, Intracellular ROS are beneficial, as they protect against invading pathogens, and ROS levels are precisely controlled by various enzymatic activities. Under pathological conditions, however, intracellular ROS levels increase due to their increased production or impaired elimination (oxidative stress). Oxidative stress causes cell damage and eventually death. ROS play an important role in the progression of several diseases including inflammation, atherosclerosis, aging, and age-related degenerative
disorders (13). We used the fluorescent probe CellROX® Deep Red Reagent (ex. 640nm, em. 665nm) to quantify oxidative stress in cells, upon interaction with oxygen species, the probe becomes brightly fluorescent. These cell-permeant dyes are weakly fluorescent while in a reduced state and exhibit photostable fluorescence upon oxidation by reactive oxygen species (ROS). We tested the antioxidant activity of three concentrations compound-A (50–75–100 μM) on THP-1 cells comparing its effect with two antioxidant molecules: Vitamin E and B6 (14), after 2 hours stimulation of cells with H₂O₂. Compound-A is able to decrease the levels of ROS in THP-1 cells after stimulation with H₂O₂, as shown in Figure 3A, D, and quantified in the histogram (Figure 3E).

ANTI-BACTERICAL ADHESIVE ACTIVITY

The antimicrobial activity of natural compounds is considered as one of their most valuable beneficial effects for the prevention and the treatment of human diseases (15-16). We investigated the antimicrobial activity of nutraceutical (Compound-C) (17) gains urinary pathogens in an in vitro model mimicking the first step in setting up of infection that is adhesion to host cells. After determination of the Minimal Inhibitory Concentration (MIC) (the lowest concentration of the compound able to inhibit the growth of selected bacterial strains) by micro-dilution broth method according to international guidelines. The in vitro ability of sub-inhibitory concentrations of the compounds under testing to inhibit adhesion of bacteria (10 Escherichia coli strains) to uroepithelial cells from healthy donors was evaluated. We did this by incubating bacteria previously grown with compound at concentrations: ½MIC and ¼MIC with uroepithelial cells, and counting adhered bacteria per cells at optical (Figure 3F) and electron microscope level (Figures 3G-I). As shown in figure the number of adhered bacteria, are reduced after strains were treated with sub-inhibitory concentrations of the tested compound.

DNA DAMAGE – COMET TEST

Natural agents, besides their roles as antioxidant, anti-inflammatory, anti-mutagenic, anti-carcinogenic and immunomodulators. Particularly, they have been also investigated for their activity in the prevention of UV-induced skin damage at the cellular and molecular levels (18). Their role in protection in different in vivo and in vitro models has been documented. It has been demonstrated that the oral feeding of hairless mice with pomegranate fruit extract or green tea (19-20), the pretreatment of human reconstructed skin with pomegranate-derived products (21), or the exposure of keratinocytes cells to delphinidin and anthocyanidin, resulted in a marked reduction of UVB-induced DNA damages (22). We analyzed the effect of two phenolic compounds Thymus vulgaris leaf extract and its major component Thymol against UVB-induced DNA damage in the human-
keratinocytes-cell line NCTC 2544, using the ‘comet assay’ (Figure 4F). The substances tested are commonly used for their expectorant, antiseptic, antispasmodic, antimicrobial, antifungal, antioxidative effects, but their genotoxic effect is still unclear. The comet test is a sensitive technique for the detection of DNA damage at the level of the single cells. Cells embedded in agarose on a microscope slide are lysed with solution containing detergent and high salt to form nucleoids of supercoiled loops of DNA. The DNA is allowed to unwind under alkaline conditions in electrophoresis buffer. Following unwinding, the DNA undergoes electrophoresis at high pH (pH>13), allowing fragmented DNA to migrate away from the nucleus. Once the slides are dried, they are stained with a DNA-specific fluorescent dye such as propidium iodide and observed by fluorescence microscopy. Undamaged cells appear as intact nucleoids (Figure 4A), damaged cells as comets (Figure 4B-E). The extent of DNA migrated from the head of the comet is directly proportional to the amount of DNA damage. Brighter and longer is the tail, higher is the level of DNA damage. Results obtained in the present study should be considered as predictive of future studies, useful to identify new plant-derivate active molecules, with antioxidant properties and UV protective effects that could be introduced in the organism by diet as food supplements or used as additional components in sunscreens.

CONCLUSION

In this paper, we described some examples of specific in vitro assays that can be used to test the biological effects of nutraceutical compounds. These assays represent only a fraction of all possible in vitro studies to evaluate the different properties of nutraceuticals; indeed, as nutraceutical development and related techniques of investigation are rapidly expanding new in vitro analytical tools are constantly required.

REFERENCES AND NOTES

6. Compound A: Xinepa
12. Compound B: Dolasik
17. Compound C: Ivuvar
Ageing population trends and the flourishing nutraceuticals market

KEYWORDS: Health, nutrition, ageing, nutraceuticals, food, beverage, pharmaceuticals.

Abstract KPMG has recently written two reports which discuss the ageing consumer and the Nutraceuticals market. Partners from KPMG discuss how Nutraceuticals companies stand to make huge profits from their older consumers if they position themselves correctly in this fast-evolving and high-growth market. Nutraceuticals is a market sitting between Food & Beverage and Pharmaceuticals and thus poses interesting challenges for this demographic.

INTRODUCTION

As much of the world faces rising rates of chronic lifestyle illness, in the UK alone, the potential cost of this to the NHS is over £10bn annually. The incentive for governments to focus on preventative care and for consumers to become more health-conscious is immense. Couple this with the fact the ageing population (65 and over) is set to double globally by 2035 to 1.1bn and then again by 2050 to 2bn, there is a fast growing market of functional foods, nutraceuticals and dietary supplements aimed towards this specific group of consumers.

In two recent reports, KPMG explores these trends and how food and pharmaceutical companies are perfectly placed to capitalise.

AGEING POPULATION TRENDS

The most recent report from KPMG (1), which focuses on the UK market, predicted that by 2020 the 65-and-over age group will outnumber under-fives for the first time. Added to this, in the last decade, spending among over 50s has grown on average 4.4% a year – faster than any other age group – and they also earn more than any other segment of the population (2). This therefore means, a greater concentration of both money and consumers at the older end of the spectrum.

Brands looking to capitalise on these trends need to also bear in mind that the older generations place far more value on quality and experience meaning they are far less discount-driven than younger shoppers. This group is also relatively brand loyal. Nestlé is a good example of a company adapting to the ‘silver’ market. Of the 1,261 consumers surveyed by KPMG and YouGov, over 70% of those aged 70+ were likely to have trouble opening packaging and have difficulty reading labels. That said, nearly 50% of those aged 20 to 60 agreed.

The Nestlé R&D team is already testing their products to identify barriers like these, including difficult packaging, which are preventing older consumers from using their products. The company has invested in a machine hand which mimics the effects of arthritis and, while it is not marketing this directly to consumers, the research is being used to make alterations to packaging which will benefit all shoppers.

NUTRACEUTICALS

When looking beyond the packaging to what’s inside the wrapper, there is growing demand from consumers for nutraceuticals products. The term nutraceuticals was coined in the 1980s to describe food products that have a medicinal benefit, and the sector has exploded in recent years to include functional foods such as vitamin-enriched products, nutritional supplements, sports drinks and medically formulated foods (3).

KPMG’s report; Nutraceuticals: The future of intelligent food (4) highlights that future superfoods could potentially tackle the underlying causes of conditions such as diabetes, obesity and cardiovascular disease, by linking diet to the human genome. It is therefore unsurprising that the global nutraceuticals market is predicted to be worth $250 billion by 2018 as a result of increased uptake by health savvy consumers.

Food companies have so far taken the lead in the nutraceuticals space, thanks to a greater understanding of nutrition, food formulation and decades of consumer research to support growth in this area. However, food companies lack the scientific resources to achieve game-changing breakthroughs with innovation tending to involve modest improvements, such as reducing sugar content.

On the other side of the coin, pharmaceutical companies are also making attempts to gain ground in the nutraceuticals...
space. For example, there have been a number of deals in the pharmaceutical and consumer healthcare sectors in recent years. In 2010 Nestlé claimed to have pioneered a new industry between food and pharma with the creation of Nestlé Health Science and Nestlé Institute of Health Sciences. More recently, in March 2015, Novartis and GSK agreed to create a consumer healthcare business through a joint venture between Novartis OTC and GSK Consumer Healthcare. While these moves have not yet led to a nutraceuticals leader being established, the direction of travel is pretty clear.

CHARACTERISTICS OF SUCCESS: DARING TO WIN

Currently, it seems neither pharmaceuticals nor food companies have all the ingredients to take the lead in the nutraceuticals market. Yet with the sector set for huge growth over the coming years it seems inevitable that, at some point, a nutraceuticals powerhouse will emerge - but what will it take to build one?

Technology
Technology is going to be crucial. The nutraceuticals market is likely to benefit from the trend of personalised medicine, and developing technology which aids this movement is key, including taking advantage of the huge amount of data that will be created by an increasingly connected healthcare system. Consumer companies have the intelligence available to personalise dietary advice, and with prudent partnerships, pharmaceutical companies may be able to personalise over-the-counter nutraceutical products to supplement consumer lifestyles.

Compliant marketing
The market for nutraceuticals is not globally uniform: countries vary in the way their regulatory systems recognise nutraceuticals – as pharmaceuticals versus food, while consumers themselves cannot agree which category these products fit into best. Successful companies will have to develop a highly nuanced global marketing campaign that can exploit economies of scale while adapting to a myriad of localised preferences, regulations and buying behaviours.

Regulation
Regulations around nutraceuticals vary from one country to another and are changing all the time. In the EU, products that claim to be a nutraceutical have to be certified by the European Food Standards Authority, and a similar system operates in Canada. However, this contrasts with the US and Japan, two markets that currently account for over half the world’s demand for nutraceuticals, where products do not have to pass stringent government tests as long as they do not claim to treat or prevent a specific disease. Manufacturers will have to work closely with governments to anticipate market opportunities that can arise when rules are altered.

Supply chain
With increasing consumer concerns about food safety, the quality of raw materials used to create nutraceutical products have come under intense scrutiny, so companies must consider carefully how to access and assure supplies of essential ingredients or flavorings. A number of recent acquisitions of food and beverage companies are aimed

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at securing stable, high quality supplies of raw materials. Vertical integration of supply chains, for example, gives greater certainty of supply, and lays a foundation for developing new uses of existing materials, or to produce new, related ingredients.

**Product strategy**
Blockbusters in this industry may be few and far between. Recently some companies have been re-balancing their product portfolios and actually abandoning product lines that do not fit their strategy. GSK are an example of this, having sold Ribena and Lucozade in 2013 to Suntory. Competitive advantage will be gained by those companies with a superior product strategy: juggling ingredients, technology and labelling to optimise the product portfolio in each country.

**Deal making**
Companies can make up for their weaknesses in certain areas by being quick and decisive corporate deal-makers. It will only take one sizable acquisition to change the dynamics of the industry, and other big companies in the field will be forced to respond. Big pharma and big consumer goods companies are not necessarily known for being nimbly entrepreneurial, so they will have to do a number of things that may go against their corporate cultures: be bold, supple and patient, all at the same time.

**REFERENCES AND NOTES**

Eve and the apple: the origin of original sin
Apple consumption and female sexual quality of life: an intriguing relationship

KEYWORDS: Apple, polyphenols, mediterranean diet, quality of life, female sexual function, FSFI questionnaire.

Abstract
The relationship between food and women’s sexual health is very intriguing. Several authors stated that the daily use of some foods or beverages, such as chocolate or red wine, are associated with a better female sexual quality of life in terms of sexual desire or lubrication. Recently, a national multicenter cross-sectional study suggest a potential relationship between regular daily apple consumption and better sexuality in our young women population, highlighting the role of some phytoestrogen such as phloridzin, a dihydrochalcone glycoside, that is contained under the apple peel. Here, we evaluate the role of daily apple consumption in the female sexual quality of life, by using a narrative review of the current literature.

INTRODUCTION
"[...] and when the woman saw that the tree was good for food, and a delight to the eyes, and to be desired to make one wise, she took of its fruit, and gave it to her husband. And their eyes were opened and they were conscious that they had no clothing and they made themselves coats of leaves stitched together” [from the Book of Genesis, 3:6-8] (Figure 1).

Female sexuality is regulated by several anatomical, neurobiological, and psychological mechanisms and their interaction characterizes the state of women’s sexual response [1]. Several studies have suggested that some foods could also have an intriguing impact on female sexual quality of life [2-3]. Mondaini et al. found that regular moderate intake of red wine is associated with higher FSFI scores for both sexual desire, lubrication and overall sexual function as compared to teetotaller status [3]. Recently, Salonia et al. showed that those women who reported eating ≥1 chocolate cubes daily have higher FSFI scores for both sexual desire and overall sexual function than women who did not report eating chocolate [2]. The authors hypothesized that reason for the impact of these foods on female sexuality may be the fact that containing polyphenols and antioxidant can stimulate peripheral vasodilitation via activation of the nitric oxide system [2-3]. During sexual arousal, in fact, the central reduction of sympathetic tone and the release of two vasodilator neurotransmitters (vasoactive intestinal peptide – VIP; nitric oxide – NO), create an increase in the blood flow to the external genitalia and vagina and promote relaxation of the smooth muscle of the cavernous sinuses in the clitoris [4].

Moreover, the vaginal capillaries of microcirculation are filled with blood and the increased hydrostatic pressure inside them forces out a plasma transudate (ultrafiltrate) in the interstitial space around the blood vessels [1]. Continued formation of this transudate fills up the interstitial space and then passes through and between the cells of vaginal epithelium to leak onto the surface wall of the vagina as the vaginal lubrication [1]. Another important factor determining normal vaginal lubrication and, generally, female sexuality, is estradiol. It is well known that post-menopausal reduction in estradiol is commonly associated with vaginal dryness, which improves with its replacement [5]. Recently, some authors have highlighted the fact that apples represent an important source of polyphenols and antioxidant in the Western diet. It has been suggested that their regular intake may result in human health benefits [6]. Moreover, it appears that apples contain phloridzin, a dihydrochalcone glycoside, a common phytoestrogen [7]. It is well known that phytoestrogens, such as phloridzin, can produce estrogen-like effects because of their structural similarity to estradiol, and can combine with estrogen receptors in mammals or humans, resulting in antiestrogenic or estrogen-like activity [8]. In this sense, daily apple intake should improve female sexual quality of life [1]. Cai et al., recently, by using a cross-sectional study in a large cohort of young sexually active women, find that daily apple use is associated with higher Female Sexual Function Index (FSFI) scores in sexually active female patients thus increasing their lubrication and overall sexual function [9]. The correlation between daily apple intake and higher FSFI scores for lubrication and overall sexual function as compared to those who did not report eating apples is intriguing [9]. The aim of the present study is to evaluate the role of apple consumption in the female sexual quality of life, by using a narrative review of the current literature.
MATERIALS AND METHODS

We conducted a search of the English-language literature from 1960 through December 2014 with use of the Medline computerized database of the US National Library of Medicine (http://www.ncbi.nlm.nih.gov/pubmed). The Medline search has been carried-out by using the following Medical Subject Headings and free text terms: apple, polyphenols were combined with the terms women health, female sexual function, FSIQ questionnaire and then limited to humans and female. Moreover, we searched reference lists of articles to identify potential additional references. All original paper and review studies on female sexual function have been considered for this review. From an initial literature search with 20 unique citations, a total of 6 articles were selected for the present review. Finally, a matched research between apple consumption and female sexual function (explored) has found 2 articles.

RESULTS AND DISCUSSION

Apple and health: compound and pharmacological proprieties

Apples have commonly been described as a healthy food. Moreover, apples are a fruit of specific interest, since they are cheap and easy to store and transport, and thus are the most frequently consumed fruit. It is well known that increasing the consumption of fruit and vegetables is a key component of obesity prevention, as their consumption reduces the risks of several diseases, such as type 2-diabetes, cardiovascular diseases or colorectal cancer (10-11). Apples, for instance, contain a lot of pharmacologically active substances such as phytoestrogens, poliphenols and antioxidants (6. 12-13). In fact, apples are large contributors of phenolic compounds in European and North American diets (6). In particular, apples, when compared with other commonly consumed fruits, had the second highest level of antioxidant activity (after cranberries) and phenolic compounds, and the highest level of free phenolic compounds (12, 14). For these proprieties, apple intake has been studied and negatively associated with lung cancer incidence (15), coronary and total mortality (16), symptoms of chronic obstructive pulmonary disease (17), and risk of thrombotic stroke (18). Moreover, it is also well known that the concentration of total phenolic compounds is much greater in the peel of apples than in the flesh (19).

Apple and sex: the phloridzin

Apples contain phloridzin, a dihydrochalcone glycoside, a common phytoestrogen (7). It is well known that phytoestrogens, such as phloridzin, can produce estrogen-like effects because of their structural similarity to estradiol, and can combine with estrogen receptors in mammalians or humans, resulting in antiestrogen or estrogen-like activity (8). In this sense, daily apple intake could improve sexual satisfaction and the couple’s quality of life. Although these findings need to be interpreted with some caution due to the limited number of published studies, daily apple intake could improve sexual quality of life in young sexually active women, thus improving sexual satisfaction and the couple’s quality of life. However, further studies will be necessary to clarify all molecular mechanisms involving the relationship between apple intake and female sexuality. Further epidemiological investigations prospectively evaluating larger cohort populations of women are needed to confirm the precise role of apple intake in the context of female sexual response. However, the present data can allow the development of future research for identifying new compounds and food supplements to use in the female sexuality recovery.

CONCLUSIONS

In conclusions apple consumption is related with better sexual quality of life in sexually active female patients. Although these findings need to be interpreted with some caution due to the limited number of published studies, daily apple intake could improve sexual quality of life in young sexually active women, thus improving sexual satisfaction and the couple’s quality of life.

REFERENCES


Sodium / potassium relationship in biofortified cucumber

KEYWORDS: Biofortification, potassium, sodium, cucumber, seed, seedlings.

Abstract Fortification policies are increasing to achieve cost savings as a way of enriching plant products. For this reason in our study we have increased potassium content in cucumber and we studied the relationship of this element with sodium. Cucumber was chosen for its easy use, including in many culinary preparations, in addition to its low calorie input.

Twenty-eight samples biofortificated with different potassium sulfate concentrations (0.014 mg / L, 1 g / L, 2 g / L and 4 g / L) were collected in three different stages from two different greenhouses, one cultivated from seed and the other one from seedlings.

We conclude potassium content of biofortified cucumbers has increased over time. After the comparison of the potassium average content in the fruits obtained in the different collections, we see that in the case of those from seedlings it is higher in the first two collection moments equaling in the last one.

INTRODUCTION

Nowadays, consumers do not only demand a high production of food but they also require nutritive products that contain the macro- and micronutrients needed by the body to improve/restore the body’s physiological functions and reduce the risk of disease (1). Policies are being developed to produce cost-saving enriched vegetables (2) and improve the efficiency of infertile soils (3). Biofortification is considered a new tool for reducing deficiencies of micronutrients (4).

There are recent studies about biofortification with different elements, such as (5), which assessed the effect of enriching seeds of different plant foods (cucumber, among others) with ZnO on germination. The results of the study revealed that the uptake of ZnO increased germination by 10% in cucumber, whereas germination decreased in other plants. Another example is (6). This study revealed that biofortification of broccoli and carrot crops with selenium –an element with important health benefits– increased Se concentrations in these food crops, which is especially relevant for Se-deficient regions.

A higher intake of potassium has beneficial effects on human health. According to (7) hypokalemia is a risk factor for heart, arterial, cerebrovascular and renal disease. Increasing the intake of potassium reduces mortality from cardiovascular disease since it reduces blood pressure and has a direct effect on the cardiovascular system (8). In accordance with (9), excessive sweating during exercise can cause an electrolyte imbalance that causes dizziness, vomiting, bloating, muscle weakness, cramps ... Recent studies conducted in different countries (10-12) confirm that the intake of sodium in the general population is above the daily recommendations, whereas the intake of potassium is insufficient.

American countries recommend a higher intake of potassium, fiber, vitamin D and calcium, and a lower intake of calories from saturated fats and simple sugars (13). Since the cost of potassium is fairly expensive, it is estimated that increasing the intake of potassium-rich products to meet daily recommendations would increase consumer costs an average of €279/year (14) in their study on the relationship between the intake of sodium and potassium and food expenditure concludes that diets with beneficial effects on cardiovascular health (those high in potassium and low in sodium) tend to be more expensive. Therefore, the intake of potassium-enriched products would be more viable from an economic point of view.

We chose cucumber for its easy handling and because it is used in many dishes worldwide; also, it is a low calorie food –12 kcal / 100g according to (15) – which is important for overweight and obese people, who require a high input of potassium.

The aim of this study was to assess whether potassium concentrations in a greenhouse-grown cucumber crop could be increased through biofortification by adding an extra amount of potassium sulfate to regular fertilizers. A secondary aim was to assess the effect of potassium biofortification on the sodium concentration of cucumber and determine whether increasing the potassium concentration of cucumber results in a reduction in the sodium concentration due to the competition between these two cations (16). Most of the studies reviewed analyzed potassium and sodium concentrations either in the
S1 and P1 abbreviations correspond to 0.014mg/L potassium sulfate, S2 and P2 to 1g/L, S3 and P4 are 2g/L; finally, S4 and P4 represent a concentration of 4g/L. The acronyms S and P indicate that the fruits were grown either from seeds (S) or from seedlings grown in seedbeds (P).

Solutions were added with PVC bottles adapted as irrigation dispensers that were placed next to the plant root system (Figure 3) and released the solution according to plant needs and soil humidity.

**Potassium and sodium determination by flame photometry**

Sodium and potassium concentrations were determined by flame photometry following the “Official Methods of Analysis of Food” (20).

**Sample preparation.** Getting ashes: 5g cucumber (in the case of samples without skin they were previously peeled with a disinfected knife) were put in a tarred quartz capsule and heated slowly in a sand bath until most of the organic substance was carbonized. Then the capsule was inserted into the muffle at 525°C for eight hours, cooled in a desiccator for 30 minutes and weighed.

**Potassium:** Appropriate sample dilution was performed with distilled water. Readings of the 766-770 nm wavelength flame photometer were performed against reference solutions. Measurements were taken three times to obtain a real value by calculating the average of the three values obtained. Potassium concentrations were calculated from the average value obtained by deducting the average value from the equation of the calibration curve taking into account the dilution made. The results reported are for the 5g samples analyzed. Conversions were performed to express results. We used a standard solution of potassium concentration of 1g/L for the calibration curve. From this solution, we prepared solutions of 0, 1, 2, 3, 5 and 7mg/L. The 0mg/L concentration was prepared only with miliQ water.

**Sodium:** Sodium concentrations were measured using the same method as that used for measuring potassium concentrations, although measurements were made at a 590 nm wavelength.

For the calibration curve a standard solution of sodium concentration 1g/L was used. Solutions of 0, 2, 5, 10, 15 and 20mg/L were prepared from the previous solution. The white solution was done with miliQ water.

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**MATERIAL AND METHODS**

The cucumber samples (Cucumis sativus L) used were Almeria type of the Valle seed variety (RZ code 24-165) developed by Rijk Zwaan and other seeds of the same variety collected from seedlings grown in seedbed and transplanted to the greenhouse in soil blocks. The greenhouses were located in the Punta Entinas-Sabinar Natural Park (Almeria). Twenty-eight samples were collected in three different stages. The first crop was collected on December 12, 2012 (Crop 1), the second on February 25, 2013 (Crop 2) and the last on April 3, 2013 (Crop 3). Of the 28 samples from each batch, fourteen had been grown from seeds in a greenhouse and the rest from seedlings grown in seedbed. We collected samples from three different plants fertilized with the same potassium sulphate concentration added to the usual fertilizer, plus two controls. Between each treated sample was left at least a white side to prevent possible contamination of the treatments, as shown in Figures 1 and 2. Samples from each treated plant were collected randomly.
RESULTS AND DISCUSSION

Student’s t test was performed for independent samples in order to determine whether there were significant differences regarding the potassium contents between the cucumbers grown from seeds shed in a greenhouse and the cucumbers grown from seeds shed in seedbed and transplanted with the root ball, the results are expressed in mg K+/100 g cucumber. We found statistically significant differences at a 95% confidence level in the average potassium concentration, which was higher in the samples from the seedlings (Table 1).

The two types of culture were analyzed separately. We assessed whether there were significant differences among the samples from the three crops. Friedman’s test was performed for comparison of related samples n>2 and revealed significant differences between the two groups over time at a 99% confidence level. Table 2 shows the results for the samples from seeds, and Table 3 displays the results for the samples from the seedlings.

Table 2 shows that average values were higher for Crop 1, which means that potassium concentrations progressively increased over time reaching an increase of 24% in Crop 2 from the first crop. As to minimum values, they also increased gradually over time. Finally, there was also an increment in maximum values for Crops 2 and 3, although the maximum value for Crop 2 was higher as compared to Crop 3. In table 3 we can see that average values were higher for Crops 2 and 3 although it was higher for Crop 2 as compared to Crop 3, which showed an increase of only 1.9%. However, the maximum potassium concentration was reached in Crop 3; in fact, standard deviation was quite significant in Crop 3; this would explain that the average potassium concentration was not higher in the samples from the seedlings in Crop 1 as it occurred in greenhouse seeds. In Crop 3 maximum values increased by 40% with respect to Crop 1. Comparing the average potassium concentration in fruits in the different crops, we see that potassium concentrations were higher in the fruits from the seedlings as compared to those from seeds in Crops 1 and 2. However, the concentration of potassium was the same for both groups in Crop 3 (Figure 4).

Next, we analyzed whether there were significant differences in potassium concentrations among samples with and without skin from the three crops. In Crop 1 we observed differences in potassium concentrations among the cucumbers from seeds with or without skin at a 95% confidence level. On the other hand, differences were found between the seedling samples with and without skin, at the same confidence level. Thus, the average concentration of potassium was higher in the samples with skin for both groups (Table 4).

In Crop 2, no significant differences were found concerning potassium concentrations among the cucumbers from seeds with or without skin at a 95% confidence level. The other hand, differences were found between the seedling samples with and without skin, at the same confidence level. Thus, the average concentration of potassium was higher in the seedling samples with skin in Crop 2 (Table 5).

In Crop 3, the initial differences between samples with and without skin had disappeared in both groups. In Crop 3, the average concentration was slightly lower in the seedling samples, as opposed to what happened in Crops 1 and 2 (Table 6).

To sum up, potassium concentrations were higher in the samples with skin of both groups in Crops 1 and 2. However, such differences between the samples with and without skin disappeared in Crop 3.
As to sodium concentrations, after verification by Student’s t test, no significant differences were observed between the seed and the seedling samples. Friedman’s test revealed significant differences in the amount of sodium absorbed over time at a 99% confidence level. Thus, in Table 7 we can see that average sodium concentrations were greater in Crops 2 and 3, which means that -as it occurred with potassium concentrations- sodium concentrations increased over time. We can also observe that maximum and minimum concentrations increased.

Initially, we expected that sodium concentrations would drop with increased potassium concentrations, as these two cations would compete for absorption. However, statistical analysis revealed that sodium concentrations did not drop with increased potassium concentrations. Nevertheless, we think that increasing potassium concentrations in cucumber may be beneficial at the metabolic level as it would mitigate the negative effects of sodium intake, as it has been shown in many studies performed in the past (21) and at present (10). The latter, which was performed on a large population sample (1,285 healthy subjects ranging from 25-64 years), reported that a high sodium intake was not associated with higher blood pressure in the subjects who had increased potassium intake through diet. Thus, this study concluded that a high intake of fresh vegetables, fruits and whole grains rich in potassium can help reduce the harmful effect of a sodium-rich diet.

CONCLUSIONS

In the light of the results obtained, we recommend greenhouse-grown cucumber biofortification with potassium sulfate without removing the skin. Since no significant differences were observed among samples in potassium concentrations, we recommend using the minimum concentration required for obtaining the same result (0.014 g/l) in order to save costs. Moreover, we recommend the biofortification of cucumber from seedlings to get a higher concentration required for obtaining the same result (0.014 g/l) in order to save costs. Additionally, we recommend using the minimum concentration of potassium during the entire culture period. In the light of the results obtained, we recommend greenhouse-grown cucumber biofortification with potassium in order to save costs. Moreover, we recommend the biofortification of cucumber from seedlings to get a higher concentration required for obtaining the same result (0.014 g/l) in order to save costs. Additionally, we recommend using the minimum concentration of potassium during the entire culture period.

REFERENCES

* Potassium Panreac Standard solution. K = 1.000 ± 0.002 g / L ICP
** Panreac Standard Sodium solution. Na = 1.000 ± 0.002 g / L AA
Abstract
The demand for animal protein is expected to increase drastically in the future because of the global growth of population and income. The increasing need for good quality protein cannot be met only with animal-based proteins without drastic adverse impacts for environment and humanity. Finding new sources of food proteins and especially plant proteins is a prerequisite to obtain sustainable and affordable foods for the growing global population. However, there are technological, nutritional and sensory challenges associated with the use of plant proteins. This article reviews the applicability of plant proteins as a food ingredient for the development of future foods and suggests strategies for improving their use in various food applications.

INTRODUCTION
The global population is forecasted to increase to 7.6-10.6 billion people by 2050. This rapid growth in combination with expected increasing welfare will lead to an increased demand for animal protein which is forecasted to increase by 52% between 2007 and 2030. Thus, additional alternative and sustainable plant protein sources with high technological, sensory and nutritional functionality are needed. Plant protein production requires less land and thus less resource intensive than that of animal protein, but plant proteins also pose challenges especially when compared to the properties of the current animal proteins.

Protein is an essential part of human nutrition. Animal proteins are “complete proteins” since they provide sufficient amounts of essential amino acids. Plant proteins are generally “incomplete proteins” as they lack one or more of the essential amino acids. Besides nutritional value, proteins contribute to the technological functionality of in food matrices, such as foam-formation, gelation, rheological and texturizing characteristics. These properties, including sensory quality, are essential in applications of plant proteins as building blocks of food structure and in design of new high-protein foods. However, most of the plant-based proteins don’t provide optimal techno-functional properties. The existing scientific knowledge is generally built around meat, dairy, or egg proteins, and of the plant proteins, only soy and wheat protein are rather well studied.

The use of soybean involves issues such as GMO which is a consumer concern. Furthermore majority of the soy protein is imported and thus interfering with the protein independency for Europe. In line with its new agricultural policy, EU is stimulating production and use of domestic plant proteins. Wheat proteins cannot be consumed by coeliac patients, and also currently suffer of the growing consumer trend of excluding gluten from the diet.

The interest in plant proteins is globally increasing rapidly, and an increasing number of commercial plant protein ingredients are already available (Table 1), with many more in pipeline.

CEREAL PROTEINS: WHEAT, RICE AND OATS
Most commonly used cereal protein is gluten which plays a key role in determining the unique quality of many cereal

<table>
<thead>
<tr>
<th>Plant Protein Ingredient</th>
<th>(%Soy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soy protein concentrate</td>
<td>65-70</td>
</tr>
<tr>
<td>Soy protein isolate</td>
<td>&gt;80</td>
</tr>
<tr>
<td>Texturized soy protein</td>
<td>60</td>
</tr>
<tr>
<td>Vital wheat gluten</td>
<td>75-80</td>
</tr>
<tr>
<td>Isolated wheat protein</td>
<td>90</td>
</tr>
<tr>
<td>Texturized wheat protein</td>
<td>Cargill, Kampfemeyer</td>
</tr>
<tr>
<td>Enzyme hydrolysed wheat</td>
<td>&gt;80</td>
</tr>
<tr>
<td>Rice protein concentrate</td>
<td>80</td>
</tr>
<tr>
<td>Rice protein isolate</td>
<td>90</td>
</tr>
<tr>
<td>Bio-Fermented rice protein</td>
<td>80</td>
</tr>
<tr>
<td>Corn Zein</td>
<td>88-96</td>
</tr>
<tr>
<td>Pea protein concentrate</td>
<td>&gt;55</td>
</tr>
<tr>
<td>Pea protein isolate</td>
<td>&gt;80</td>
</tr>
<tr>
<td>Fababean protein concentrate</td>
<td>60</td>
</tr>
<tr>
<td>Canola protein isolate</td>
<td>50</td>
</tr>
<tr>
<td>Hydrolysed canola protein</td>
<td>83</td>
</tr>
<tr>
<td>Sunflower seed protein</td>
<td>45-55</td>
</tr>
<tr>
<td>Potato protein isolate</td>
<td>&gt;92</td>
</tr>
</tbody>
</table>

Table 1. Major commercially available plant protein ingredients.
PULSES AS SOURCES OF PROTEIN

Pulses are an excellent, low cost source of protein, as well as dietary fibre, minerals and vitamins (Table 2).

<table>
<thead>
<tr>
<th>Crops</th>
<th>Protein content (% dm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Common beans</td>
<td>21-24</td>
</tr>
<tr>
<td>Cowpea</td>
<td>23-24</td>
</tr>
<tr>
<td>Chickpea</td>
<td>19</td>
</tr>
<tr>
<td>Pigeon pea</td>
<td>22</td>
</tr>
<tr>
<td>Lentils</td>
<td>26</td>
</tr>
<tr>
<td>Faba beans</td>
<td>28-35</td>
</tr>
<tr>
<td>Soybeans</td>
<td>36</td>
</tr>
<tr>
<td>Wheat</td>
<td>10-14</td>
</tr>
<tr>
<td>Maize</td>
<td>10</td>
</tr>
<tr>
<td>Barley</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 2. Protein content of major pulse crops and cereals.

They are a recognised protein source in human nutrition already now especially in Asian countries, and can offer an interesting alternative and addition to animal protein when used in new applications. Legumes, particularly beans, have been called as the “poor man’s meat” due to their high protein content, ranging from 19 to 36%, and excellent nutritional profile and yet low cost. Faba beans, for example, have good amino acid composition, and are rich in essential amino acids isoleucine, leucine, lysine, phenylalanine, threonine and valine. When combined with cereals (rich in sulphur-containing amino acids) they offer a balanced plant protein source with nutritionally adequate amino acid composition. Gluten-free fermented faba bean flour could be applied to obtain high protein bread recipes together with corn starch (Figure 2).

Among the pulses, soybeans have been in the centre during the past 15-20 years not only due to their high protein content but also as a source of a group of phytochemicals called isoflavones. About 85 percent of the world’s soybeans are processed, or “crushed,” to yield oil and meal, 98 percent of the latter going to animal feed. 

In current grain and seed processing, side streams are often generated which are under-exploited or even wasted. Many of these are used as feed especially because of their high protein content. Valorisation of side streams in the form of upgraded protein ingredients will benefit food industry by development of sustainable protein ingredients alternative to animal derived ones. Processing of cereal grains for starch or bioethanol production generates substantial volumes of co-streams rich in protein. Wheat bran, 167 million tons of which is produced annually, is currently used as cattle feed, biofuel production or even discarded totally. In wheat the bran fraction is around 14–19% of the whole kernel, and contains 8.3–19.3% protein. The aleurone layer which makes half of the wheat bran is rich in protein and has superior amino acid composition compared to wheat endosperm (low in methionine, sulphur-rich proteins and cysteine; high in essential amino acids valine, threonine and especially lysine). Rice bran is undervalued side stream of rice starch or milling industries. Rice bran is generally not consumed as food because of its high fibre content, possible hull contamination and rancidity issues due to lipase activity. Thus, it is mainly used for feed or fuel. Ten percent of the rice kernel is rice bran which contains from 12% (native bran) to 20% (defatted bran) protein. In rice bran, water soluble albumins comprise the major protein fraction (37% of bran proteins) together with globulins (36% of bran proteins) which may render rice bran proteins with good functional properties compared to other cereal proteins as well as rice endosperm proteins in water-rich food matrices.

Oats are an important minor crop worldwide with the global production of 21 million tons per year, 62% of which is in EU. Use of oats in food is still limited, and finding new uses would diversify the consumption of grains in Europe, now dominated by wheat. Concentration of oat beta-glucan in a dry fractionation process makes also available a stream rich in oat protein. Oats is exceptional within cereal crops with respect to nutritional value because of higher lysine and basic amino acid content. Oat protein has also potential because it can be in many countries used in gluten-free diet.
The rest is consumed in various forms of protein ingredients. Soy concentrates (70% protein) and isolates (90% protein) are obtained from defatted soybean meal through a series of acidic or ethanol extractions, rendering them variable functional properties as the conditions might cause protein denaturation. Soy concentrates are used for bakery products whereas soy isolates are used in meat and dairy foods where emulsifying, thickening and gelling properties are crucial (8).

Pea protein, extracted from yellow pea, is becoming popular and after soybean it is the second most used pulse protein. It is one of the few plant protein ingredients without bitter taste. However, the expensive solvent extraction method used for pea protein is complex. Pea protein concentrate (51-55% protein) and isolate (85% protein) applications include meat, fish, processed foods, soups, sauces, baked goods cereals, snacks, nutraceuticals, sports, and clinical nutrition.

Dry beans (e.g., white, black, navy, kidney, pinto, fava and field bean) are high in fibre, calcium, and iron, but also a great source of protein (15-25% wb) and soluble fibre. Dry bean proteins remain largely underexploited. They include the water-soluble (albumins) and dilute salt-soluble (globulin) storage proteins and some of the metabolic proteins such as the enzyme inhibitors and lectins. Most bean proteins have acidic isoelectric points because of large amounts of glutamic and asparagic acids, have water holding capacity varying 5-6 times of their own weight and oil holding capacity < 5 g oil/g (9). For gel formation from bean proteins higher protein concentration (10 to 20% w/v) and temperature (> 70°C for several plant proteins compared with 4°C for gelatin) is required compared to animal proteins. Several dry bean storage proteins have excellent emulsion properties, both emulsion capacity and stability, and therefore have been used successfully in many countries (e.g., India, Egypt, Turkey, China) to prepare local foods.

Lupine, an ancient leguminous seed originated more than 3500-4000 years ago back, is being increasingly used in food formulations due to its nutritional and functional properties (10). The protein and dietary fibre content in lupine accounted for 29-34 and 50-40 g/100 g d.m depending on the variety. Lupine protein can replace egg proteins and their properties (10 to 20% w/v) and temperature (> 70°C for several plant proteins compared with 4°C for gelatin) is required compared to animal proteins. Several dry bean storage proteins have excellent emulsion properties, both emulsion capacity and stability, and therefore have been used successfully in many countries (e.g., India, Egypt, Turkey, China) to prepare local foods.

FUNCTIONALITY OF PLANT PROTEIN INGREDIENTS

Compared with animal proteins, notably dairy and meat proteins, research on plant proteins, with the exception of soy proteins, has so far been limited. Solubility or dispersability in the form of stable colloidal aggregates is one of the main requirements for functionality of proteins as emulsifying, foaming or gel forming agents. Plant proteins generally show low solubility (or dispersability) at pH values relevant to most foods. Furthermore, processes such as oil extraction or high temperature treatments for enzyme inactivation may induce denaturation which further decreases solubility. These physico chemical properties restrict full exploitation of plant proteins in different foods. Despite the low solubility of plant proteins, the so-called soluble aggregates (colloidal particles) at certain environmental conditions (pH, ionic environment) have shown good surface active properties. For example, soluble aggregates of oat globulins were reported to lower surface tension at air-water interface which was comparable to milk proteins (16). It was recently shown that colloidal zein (corn protein) particles (~ 70 nm) had good wetting properties at the oil-water interface at various environmental conditions rendering them good inherent surface activity (17). In fact, the remarkable capability of particles to stabilize foams and emulsions (pickering emulsions) is well documented (18-19).

For solid food applications such as bread, the addition of isolated or concentrated plant proteins in the formulation impacts on the rheological properties of the dough as well as the characteristics of the final products. In bread making, the enrichment with isolated proteins (e.g., lupine, sesame, barley) increases water absorption of the dough and makes it less stable and less resistant to prolonged kneading (20-22).
As these proteins are not as elastic as gluten, they do not form a network and thus do not allow cell expansion, so the crumb appears more compact. Harder crumb is probably a result of the thickening of the crumb walls surrounding the air cells and strengthening of the crumb structure by the non-gluten-forming protein particles [22]. However, different types of protein isolates, depending on their origin, extraction methods and pre-treatments, have different effects on the quality of baked products [23].

STRATEGIES FOR MODIFICATION OF FUNCTIONALITY

Degree of purity is important for the functionality of a protein ingredient. Protein-carbohydrate interactions at some conditions might even have a positive impact on improving the techno-functional properties. The separation of protein by dry fractionation techniques depends greatly on the interactions of cell walls, protein and starch components in the plant cell matrix. Milling is usually needed to enhance the dissociation between starch, protein and fibre rich particles, and it is important to selectively break up plant cell components without causing additional damage to other compounds [24]. The yield and purity of protein-rich fractions during the separation of protein from the other cell components depends strongly on the milling operation and the milling quality.

The applicability of plant proteins in foods can also be improved by modification of the protein itself. Potential food-compatible technologies for improving the technological functionality of proteins include complete or limited hydrolysis, protein cross-linking and protein functionalization by enzymatic means, shear-induced processing, microfluidization, complexation with polysaccharides or with other protein [16, 25]. Enzymatic hydrolysis improved the water and fat absorption ability, solubility, emulsifying and foaming properties but not foam stability of rice bran proteins [26].

FUTURE PROSPECTS

As animal protein sources become limited, the use of more plant protein is inevitable. The offering of plant protein ingredients will increase rapidly. Building on the existing protein ingredients, there is vast activity to find new sources of plant proteins by valorising side streams of current grain and seed processes, but also through introduction of new protein crops with good nutritional and technological properties. Plant proteins have several challenges, related to their limited solubility and functionality, and delivery of essential amino acids. The former can be overcome by modifications at different structural level, and much research is warranted here. The latter can be tackled by establishing a variety of proteins sources to be combined in the diet. Little is as yet known about the digestibility of plant proteins, and how processing changes it. Taste aspects are the major consumer concern, and flavour and sensory design will be an important part of future work.

REFERENCES
25. Nordlund E., Katina K., Aura A., et al. Changes in bran structure by shearing-induced processing, microfluidization, complexation with polysaccharides or with other protein (22). However, different types of protein隔离, depending on their origin, extraction methods and pre-treatments, have different effects on the quality of baked products (23).
Sensory and physico-chemical evaluation of commercial coffees consumed in Banja Luka (Bosnia and Herzegovina) – Part 2


KEYWORDS: traditional black coffee quality, descriptive sensory analysis.

Abstract The objective of this study was to evaluate the quality and establish the sensory profile of five traditional coffee beverages prepared with commercial roasted ground coffee blends of known trademarks available on the market in Banja Luka [Bosnia and Herzegovina] in 2014. Descriptive sensory analysis was used to identify and evaluate properties that influence coffee beverage preference. Caffeine, fat and fatty acids content and CIEL*a*b* colour parameters of roasted ground coffee samples were analysed. Differences between analysed coffee beverages were determined in sensory properties colour, bitterness, acidity and sweetness and aroma attributes. Consumers preferred dark brown with orange-reddish hue coffee beverages, with moderately expressed coffee-like and less pronounced roasted, caramel-like and nutty aromas, and moderately pronounced bitterness, less pronounced acidity, and mild sweetness. Cereals and roasty aromas, as less desirable were moderately expressed. Burnt, dusty and earthy aromas were undesirable and less pronounced in coffee beverage.

INTRODUCTION

Roasted ground coffee has a long tradition of preparing and consuming worldwide, as a warm beverage, due to its characteristic sensory properties. Freshly brewed coffee has been associated with pleasure and enjoyment by stimulating our senses, from the alluring smell of aroma volatiles to the first sip inducing a well-balanced taste impression, centring on bitterness and acidity (1-2). It is usually prepared using varieties of Arabica and Robusta species, or their blends in known trademarks commercially available on the market (3-5). The economic success of the food processing enterprises in a competitive environment depends on their capability to offer food products that are tuned to the wishes of consumers, to satisfy their expectations and so increase productivity and competitiveness (6). The quality and acceptability of coffee is correlated with its sensory properties. The descriptive sensory analysis may be used for quality control and comparative assessment of samples, to determine sensory properties, which impact on acceptance or rejection of the coffee beverage. The quality of coffee beverage depends on the green and roasted coffee beans composition, which are affected by conditions of drying, storage, roasting and grinding and method of the coffee beverage preparation (7-8). Chemical composition, as caffeine, fat and fatty acids content in the coffee bean depends on the coffee variety (5). The integrity of the beans is important to keep coffee fresh and to avoid the aging, caused by hydrolysis and oxidation of triacylglycerols (9). Among the most important fatty acids for coffee freshness are oleic ([C18:1n-9], linoleic ([C18:2n-6] and linolenic ([C18:3n-3]) acids (10). Quality of roasted coffee cannot be evaluated only by chemical analysis results (11), because of the high importance of sensory properties of the coffee beverage prepared of it. Aroma and taste of coffee are determined by a complex mix of components, especially those formed during the beans roasting, and they have always been recognized as important attributes for their sensory quality (12). The combined sensation of aroma, taste and mouth feel interact in flavour forming (13). The first sip of a warm coffee should leave an impression of a well-balanced taste centred on pleasant bitterness and acidity that characterize this product (1). The objective of this study was to evaluate the quality and establish sensory profile of five traditional coffee beverages prepared from commercial roasted ground coffee blends of known trademarks, available on the market in Banja Luka [Bosnia and Herzegovina] in 2014. Descriptive sensory analysis was used to evaluate selected sensory properties that influence coffee beverage preference. Caffeine and fat content, fatty acids composition and CIEL*a*b* colour parameters of roasted ground coffee samples were analysed.
MATERIAL AND METHODS

Coffee samples

Five commercial samples of roasted ground coffee from different producers randomly selected, purchased from local market in Banja Luka (BA) in 2014, were used for experiment. Each group of samples (5x200g coffee for each sample) was from the same production batch. The compared samples had approximately uniform shelf life, and were properly packed and labelled. All coffee samples were blends of Arabica and Robusta.

Descriptive sensory analysis

Descriptive sensory analysis of coffee beverage samples were conducted in the Laboratory for sensory analysis of foods, designed according to the ISO 8589:2007 (14). The coffee samples were ranked in previous research (2), based on overall quality preference from 1st place the sample with most, to 5th place, the sample with the least pleasant quality (15). The panel for the coffee beverages descriptive analysis was formed by 32 selected and trained assessors, and they worked individually in the booths (16-19). Each assessor participated in two sessions, and 3 samples per session were presented, according to the balanced – incomplete – 3 of 5 block design (15, 20). Each sample was analysed 21 times. Bottled drinking water and neutral crackers were used for the panelist’s receptors regeneration.

Preparation and presentation of coffee samples

Coffee beverage samples were prepared according to the ISO 6668:2008 (21). The water was heated to boiling point (90°C), poured into the special coffee pot containing coffee, and heated to boiling, making coffee beverage with rich foam. The beverage was removed from the hotplate, left the coffee to precipitate partially in coffee pot (3-5 minutes) and poured into a thermos bottle [pre-heated with hot water]. Each sample (~50 ml/cup) was served warm (~55°C) in white arkopal coffee cups (130 ml volume) pre-heated to 50°C. Samples were prepared shortly before the sensory analysis and labelled with a 3-digit code.

Each assessor was presented a specific subset of the 3 of 5 analysed samples in a randomized order, according to the balanced – incomplete block design (15, 20).

Chemical and physical attributes

Caffeine was extracted from roasted ground coffee with dichloromethane. The absorbance of the extract was determined by UV/Vis spectrophotometer (PerkinElmer Lambda 25) at the 274.7 nm wavelength (22). Fat content was determined by the Soxhlet method (23). To determine the fatty acids content in coffee fat, samples were derivatized according to the methodology of Kravi et al. (24). The analyses of fatty acid methyl esters were performed on a Hewlett-Packard (HP) 5890 gas chromatograph coupled with a HP 5971A mass spectrometer detector. The results of all analyses were expressed as the mean value of three measurements.

Colour parameters (CIEL*a*b*), as indicators of lightness, redness and yellowness were determined using a tristimulus colorimeter (Minolta CP410) with standard illumination D65, and colorimetric normal observer angle of 2°. The results were expressed as the mean value of five measurements.

Statistical analysis

The data obtained by descriptive sensory analysis were statistically analysed using one-way analysis of variance (ANOVA), and t-test was performed to determine if statistically significant difference between the mean values of analysed parameters at the p<0.05 confidence level exist. Descriptive Statistical Analysis of all data for caffeine, fat, fatty acids content and colour parameters of coffee samples were realised by Microsoft Office Excel 2007 and presented as mean values ± standard deviations.

RESULTS AND DISCUSSION

Colour of coffee depends on the degree of roasting. Comparing visually assessed brown colour and hue of the evaluated coffee beverage samples, it could be concluded that the basic colour was dark brown. Orange shade was identified in all coffee samples with variation in intensity and proportion of yellowish and reddish hue. The colour of samples G and D was dark brown with orange and discreet yellow hue. The samples B and P were dark brown with an orange hue and with slight reddish tones. Sample F was dark brown with a striking orange shade.

The sample B was the best evaluated in preference test (2). Results of descriptive analysis showed that sample B had the highest intensity of the coffee-like aroma (Table 1), as one of the most appreciated in coffee beverages (25). Statistically significant differences [p<0.05] were found between sample B and other coffee beverages. Caramel-like aroma was less pronounced and identified only in sample B, and if had less pronounced nutty aroma, significantly different [p<0.05] comparing with other samples. Roasted aroma was less pronounced in sample B similar as in samples F, P and D. Cereal and malty aromas were moderately expressed, and less burnt, dusty and earthy. Sample F was on the second and sample G was on the third place in preference test. Pleasant aroma of roasted ground coffee and cereal and malty aromas were characteristic attributes of sample F. Less desirable burnt and dusty aromas
were moderately expressed and coffee-like and nutty less. Grassy aroma was identified only in sample F. Cereal and malty aromas were the most expressed in sample G, but burnt aroma was moderately expressed and significantly different (p<0.05) of other samples. Dusty aroma, associated with aroma of toasted coffee was moderately expressed. Less pronounced were earthy and pleasant roasted and nutty aromas. Samples D and P had the least preferred aroma. The dusty aroma was expressed in all samples, but the highest was in sample D. Pleasant nutty aroma was moderately expressed and dominated in the sample P comparing to the other samples. Undesirable drugs-like aroma were identified only in samples P and D (Table 1). Earthy and drugs-like aromas had the highest intensity in sample P coffee beverage, impacting on its lowest preference (2).

The sensory analysis of bitterness, acidity and sweetness in traditional coffee beverages showed different intensity. Bitterness of coffee beverage is highly related to the roasting degree and arises from many compounds, partly from caffeine (8). Bitterness intensity was moderately expressed in samples B, P and F, and they were significantly different (p<0.05) of sample G. Acidity of the coffee samples was characterized as moderately expressed in samples G, D and P, and with slightly lower intensity in samples B and F. The relatively high acidity was in samples G, P and D. A mild note of sweetness was identified in all samples (Table 1). Descriptive analysis crossed with preference test showed that coffee beverages samples B and F, with the moderately expressed bitterness, less pronounced acidity and mild sweetness were the favourite. Sample G was on the third place, with moderately expressed acidity, less bitter and with the highest sweetness. Sample P had more expressed bitterness than sample D, and both had moderately expressed acidity, mild sweetness (Table 1) and the lowest level of overall quality impression (2).

Caffeine, fats and fatty acids content in roasted ground coffee depends on the used varieties and their relation in coffee blends (5). The main fatty acids of six analysed were linoleic and palmitic acids (Table 2). Similar results of analyses for linoleic and palmitic acids reported Vila et al. (26). Other fatty acids content, identified in coffee samples, had lower average values. Similar results were presented in literature (4, 26-27) for content of fatty acids in roasted Arabica and Robusta coffee. CIEL*a*b* colour parameters for roasted ground coffee samples were measured to characterize it (Table 2). Measured values for L* (lightness) and b* (yellowness) were the highest on sample P, the highest values for a* (redness) had, as follows: samples D, F, B, G and F. Identified difference in a* values could be related to the raw coffee colour, which depends on its variety and processing (28-29). Producers should identify which coffee products consumers like and dislike, and why. Quality and preference analyses techniques are able to solve that problem, and descriptive sensory analysis could evaluate the product properties and identify attributes related to the preferences. Producers should incorporate the data in the specification as important quality parameters for roasted coffee production and quality control, and offer in the market roasted ground coffees which has quality harmonized with the expectations of consumers on the target market.

CONCLUSION

Established sensory profile for analysed coffee beverage identify expected and preferred quality characteristic on target market. Consumers prefer dark brown, with orange-redish hue coffee beverages with moderately expressed coffee-like and less pronounced roasted, caramel-like and nutty aromas. They like moderately expressed bitterness, less pronounced acidity and mild sweetness in coffee beverages. Cereal and

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**Table 1.** Results of descriptive sensory analysis of coffee beverage prepared from five commercial samples of roasted ground coffees from local market in Banja Luka (BA) in 2014.

<table>
<thead>
<tr>
<th>Evaluated sensory attribute</th>
<th>G</th>
<th>B</th>
<th>F</th>
<th>P</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coffee-like</td>
<td>2.00±0.37*</td>
<td>2.66±0.47**</td>
<td>2.17±0.33**</td>
<td>2.25±0.24**</td>
<td>2.43±0.32**</td>
</tr>
<tr>
<td>Roasted (roasted ground coffee)</td>
<td>2.78±0.44**</td>
<td>2.50±0.32**</td>
<td>2.43±0.38**</td>
<td>2.55±0.37**</td>
<td>2.37±0.41**</td>
</tr>
<tr>
<td>Choice-like</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Carmel-like</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Flowery</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fruity</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cereal and malty</td>
<td>3.33±0.36*</td>
<td>3.00±0.42**</td>
<td>2.75±0.38**</td>
<td>1.75±0.35**</td>
<td>-</td>
</tr>
<tr>
<td>Nutty</td>
<td>2.67±0.26*</td>
<td>2.00±0.25**</td>
<td>2.25±0.42**</td>
<td>2.55±0.37**</td>
<td>2.80±0.36**</td>
</tr>
<tr>
<td>Grassy</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3.00±0.37**</td>
</tr>
<tr>
<td>Earthy</td>
<td>2.50±0.36*</td>
<td>2.25±0.37**</td>
<td>-</td>
<td>3.20±0.29**</td>
<td>2.18±0.35**</td>
</tr>
<tr>
<td>Dusty</td>
<td>3.10±0.57*</td>
<td>2.79±0.41**</td>
<td>3.04±0.31**</td>
<td>3.00±0.41**</td>
<td>3.11±0.41**</td>
</tr>
<tr>
<td>Burnt</td>
<td>3.14±0.24*</td>
<td>2.63±0.34**</td>
<td>3.38±0.40**</td>
<td>2.95±0.36**</td>
<td>2.50±0.40**</td>
</tr>
<tr>
<td>Smoky</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Drugs-like</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3.35±0.48**</td>
</tr>
<tr>
<td>Bitterness</td>
<td>2.83±0.27*</td>
<td>3.26±0.47**</td>
<td>3.18±0.34**</td>
<td>3.26±0.37**</td>
<td>2.92±0.45**</td>
</tr>
<tr>
<td>Acidity</td>
<td>3.05±0.42*</td>
<td>2.63±0.46**</td>
<td>2.71±0.37**</td>
<td>2.97±0.44**</td>
<td>3.00±0.43**</td>
</tr>
<tr>
<td>Sweetness</td>
<td>1.14±0.24*</td>
<td>1.00±0.26</td>
<td>1.03±0.30</td>
<td>1.00±0.26</td>
<td>1.00±0.0</td>
</tr>
</tbody>
</table>

*Values for expressed intensity were defined on scale from 5 (intensively) to 1 (mildly). - Statistically significant difference for mean values considered at the p<0.05 confidence level. - Values with the different superscript (ab, cd, ef, gh) within a row are significantly different (p<0.05).

**Table 2.** Caffeine, fat, fatty acids content and colour parameters (CIEL*a*b*) for five commercial samples of roasted ground coffees from local market in Banja Luka (BA) in 2014.

<table>
<thead>
<tr>
<th>Analysed parameters</th>
<th>P</th>
<th>D</th>
<th>F</th>
<th>G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caffeine (%)</td>
<td>2.01±0.08</td>
<td>1.83±0.17</td>
<td>1.94±0.14</td>
<td>2.01±0.09</td>
</tr>
<tr>
<td>Fat (%)</td>
<td>13.52±0.69</td>
<td>15.17±0.11</td>
<td>14.52±0.12</td>
<td>19.20±0.23</td>
</tr>
<tr>
<td>Palmitic (16:0 %)</td>
<td>32.60±0.80</td>
<td>35.30±0.07</td>
<td>31.61±0.68</td>
<td>34.12±0.78</td>
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<tr>
<td>Stearic (18:0 %)</td>
<td>7.86±0.61</td>
<td>7.46±0.29</td>
<td>8.76±0.71</td>
<td>7.60±0.24</td>
</tr>
<tr>
<td>Oleic (18:1n9 %)</td>
<td>12.75±0.72</td>
<td>12.15±0.15</td>
<td>10.70±0.26</td>
<td>11.60±0.90</td>
</tr>
<tr>
<td>Linoleic (18:2n6 %)</td>
<td>42.10±0.70</td>
<td>41.10±0.59</td>
<td>43.20±0.94</td>
<td>43.31±0.51</td>
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<tr>
<td>Linolenic (18:3n6 %)</td>
<td>2.1±0.02</td>
<td>2.1±0.14</td>
<td>2.6±0.18</td>
<td>1.8±0.01</td>
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<tr>
<td>Arachidic (20:0 %)</td>
<td>2.1±0.14</td>
<td>2.0±0.24</td>
<td>3.3±0.15</td>
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<tr>
<td>Light intensity (L*)</td>
<td>42.72±0.09</td>
<td>42.53±0.04</td>
<td>41.26±0.09</td>
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<tr>
<td>Share of red (a*)</td>
<td>8.24±0.21</td>
<td>8.46±0.05</td>
<td>7.84±0.10</td>
<td>8.02±0.11</td>
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<tr>
<td>Share of yellow (b*)</td>
<td>4.62±0.32</td>
<td>4.41±0.11</td>
<td>3.90±0.15</td>
<td>4.20±0.16</td>
</tr>
</tbody>
</table>

*The content of each fatty acid is expressed as mass percentage in total fat content.
The coffee companies could use results of the study for standardisation of ground roasted coffee quality and fulfill consumers’ expectations on target market.

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Salvia jurisicii Košanin and Salvia amplexicaulis Lam. Chemical composition of the essential oils

KEYWORDS: Salvia, essential oil, GC-FID, GC-MS, sesquiterpenes, spathulenol.

Abstract Numerous Salvia species were proven to be biologically active due to their chemical composition, and also promising in searching for new medicines, cosmetics, or food ingredients of natural origin. The chemical composition of the essential oils of endemic species Salvia jurisicii as well as of S. amplexicaulis growing wild in Macedonia was analyzed using GC-FID and GC-MS. The main class of compounds in S. jurisicii oil were sesquiterpenes, consisting of spathulenol (12.3%), 3-carenetene (7.0%), n-germacrene (5.1%), germacrene (4.8%) and thujapenc-2-ol (4.5%) as dominant components. Sesquiterpenes were also the most abundant class in S. amplexicaulis oil, with caryophyllene oxide (11.3%), germacrene D (7.8%), thujapenc-2-ol (7.0%), germacrene-4 (15), 5,10,14-tetra-1-ol (5.4%), α-cadinol (5.3%) and spathulenol (5.1%) as the most abundant components.

INTRODUCTION

Essential oils have been widely used since the middle ages for bactericidal, antiviral, fungicidal, antiparasitic, insecticidal, medicinal and cosmetic applications, especially nowadays in sanitary, pharmaceutical, cosmetic, food and agricultural industries [1,2]. It contains a variety of volatile molecules such as terpenes and terpenoids, phenol-derivative aromatic and aliphatic components [2]. Pharmacological investigations showed its antioxidant, antibacterial, antifungal, antiviral, cytotoxic, antinflammatory and tumorigenesis-preventing and other activities [2-5] as well as ecological significance such as pest-toxic and repellent activity [3]. Salvia jurisicii Košanin (Baikan sage, Jurisic’s cuteral sage) is herbaceous perennial endemic to the southern regions of the former Yugoslavia (Macedonia) [6]. Our previous study of S. jurisicii herb comprised antioxidative activity, total phenolic and flavonoid content of different extracts [7]. To the best of our knowledge, data on chemical composition or biological effects of its essential oil have not been reported previously.

Salvia amplexicaulis lam. (stem clasping violet sage) is herbaceous perennial that is native to southeastern Europe (7). Our previous investigations showed strong antioxidative activity of S. amplexicaulis extracts correlated to high amount of phenolics and flavonoids (8). It has been reported on high amounts of sesquiterpenes in essential oil of S. amplexicaulis, collected from mountainous region of southeastern Serbia (9). Also, antimicrobial activity of the essential oil of S. amplexicaulis growing in Serbia was reported, and better inhibition of the growth of Candida albicans and Gram-positive bacteria was noticed comparing to Gram-negative bacteria [5].

The aim of this study was to characterize chemical composition of the essential oils of Salvia jurisicii, endemic species of great horticultural interest, which to the best of our knowledge has not yet been investigated previously, and Salvia amplexicaulis, which has not been investigated from its natural localities in Macedonia. This study has not been performed with the ambition to characterize population chemical properties, but to anchor early stage chemical data on two scarcely observed Salvia species.

MATERIAL AND METHODS

Plant material

Aerial parts of Salvia jurisicii Košanin were collected in natural habitat, near city of Stip, while S. amplexicaulis plant material was collected in locality Pietzar (Macedonia). Both species were collected during period of full flowering stage, in July 2012. Due to the lack of plant material, and preservation legends on these species, plant material was collected in bulk from about 20-50 plants (approximately 500 g. depending of species) per site, thus creating a unique sample per species for further analysis. Plant material was air-dried and kept in shadow at room temperature for further processing. Voucher specimens were deposited in the Herbarium of the Institute of Botany and Botanical Garden “Jevremovac”, Faculty of Biology, University of Belgrade (BEUJ; voucher No. 16674 and BEOU; voucher No. 16673, respectively).
Essential oil isolation

Air-dried aerial parts (100g) of Salvia jurisicii and S. amplexicaulis, randomly taken from whole collected sample of each species, were grounded. Volatile oils were obtained by hydrodistillation using a Clevenger type apparatus during 3h (10).

Essential oil analysis

Qualitative and quantitative analysis was carried out using GC-FID and GC-MS. In the first instance model HP-5890 Series II gas chromatograph equipped with a split-splitless injector, HP-5 capillary column (25 m x 0.32 mm, film thickness 0.52 μm) and a flame ionization detector (FID), was employed. Hydrogen was used as carrier gas (1 ml min⁻¹). The injector was heated at 250 °C, the detector at 300 °C, while the column temperature was linearly programmed from 40 to 260°C (4 °C min⁻¹). GC–MS analyses was carried out under almost the same analytical conditions, using HP G 1800C Series II GCD analytical system, equipped with HP-5MS column (30 m x 0.25 mm x 0.25 μm). Helium was used as carrier gas. The transfer line (MSD) was heated at 260 °C. The EI mass spectra (70 eV) were acquired in the scan mode in the m/z range 40–400. In each case 1 μl of sample solution in ethanol (10 μl ml⁻¹) was injected in split mode (1:30). The identification of constituents was performed by matching their mass spectra and retention indices with those obtained from authentic samples and/or NIST/Wiley spectra libraries, using different types of search (PBM/NIST/AMDIS) and available literature data (11,12). The percentage compositions were obtained from electronic integration measurements using flame ionization detection (FID; 250 °C).

RESULTS AND DISCUSSION

The aerial parts of S. jurisicii yielded less than 0.01% of the essential oil (0.01 g EO/g of dry plant material), which chemical composition is presented in Table 1. Seventy-three compounds were identified representing 95.5% of the oil. Oxygenated sesquiterpenes (51.2%) and sesquiterpene hydrocarbons (23.6%) were dominant classes of the terpenes. Monoterpene hydrocarbons were present in lesser amount (4.0%) as well as oxygenated monoterpenes (3.6%), while oxygenated diterpenes were found in small percent (1.9%). Among hydrocarbons, aliphatic fraction was more abundant (6.5%) comparing to aromatic (4.6%). Spathulenol (12.3%), β-bourbonene (7.0%), n-nonanal (5.1%), γ-muurolene (4.8%) and thujopsan-2-α-ol (4.5%) were characterized as main components.

The aerial parts of S. amplexicaulis also yielded less than 0.01% of the essential oil (0.01 g EO/g of dry plant material). Chemical composition of the essential oil is presented in Table 2. Fifty-two compounds, representing 97.5% of the total oil, were identified. Oxygenated sesquiterpenes and

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### Table 1. Chemical composition of Salvia jurisicii essential oil.

<table>
<thead>
<tr>
<th>Compound</th>
<th>RI</th>
<th>%</th>
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<td>1-sabinene</td>
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<td>β-pinene</td>
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<td>leole-7,13-dien-15-ol</td>
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</table>

**Grouped components**

- Aliphatic hydrocarbons: 6.5
- Aromatic hydrocarbons: 4.6
- Monoterpe hydrocarbons: 4.0
- Oxygenated monoterpenes: 3.6
- Sesquiterpene hydrocarbons: 23.6
- Oxygenated sesquiterpenes: 51.2
- Diterpene hydrocarbons: 0.0
- Oxygenated diterpenes: 1.9

| Total identified | 95.5 |

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* Retention index relative to n-alkanes on HP-5 capillary column.
* Tentative identification
n.i., not identified

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(10) Hydrodistillation refers to the method of essential oil extraction.

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Table 1. Chemical composition of Salvia jurisicii essential oil.
sesquiterpene hydrocarbons were the most abundant classes of the terpenes represented with 56.5% and 34.6%, respectively.

Monoterpane hydrocarbons were present only in oxygenated form (5.6%). The major constituents of this oil were carophyllene oxide (11.3%), germacrene D (7.8%), thujopsone-2-ol (7.0%), germacr-4(15)-5(10)-14-tren-1-ol (5.4%), α-cadinol (5.3%), and spathulenol (5.1%).

Obtained results showed that oils of both studied species were rich in oxygenated sesquiterpenes (more than 50% of total oil). Sesquiterpenes were also previously recognized as predominant class in Salvia oils with different main compounds, such as viridiflorol in S. officinalis [4], β-caryophyllene, germacrene B, caryophyllene oxide, α-sclareol, germacrene D in S. nemorosa and (E)-β-polignene, α-guajene, germacrene D in S. verticillata [13], viridiflorol, manool and α-humulene in S. argentea [14], spathulenol in S. reflexa and caryophyllene oxide in S. nemorosa and S. glutinosa [15], germacrene B, β-caryophyllene, spathulenol and α-humulene in S. chinensis [16], viridiflorol, manool, α-thujene and α-humulene in S. argentea [17].

Our findings are in accordance with previous studies of S. amplexicaulis collected from two different locations in Serbia, where essential oil was characterized by high quantity of sesquiterpenes, with germacrene D, viridiflorol, caryophyllene oxide and α-caryophyllene as dominant components (5.9). Sesquiterpene alcohol spathulenol was dominant component of S. juricis oil (12.3%) and one of dominant components of S. amplexicaulis oil (5.1%). Several studies reported that spathulenol was very potent spasmyloytic agent [18], powerful against multidrug resistance cancer cells [19] and very strong immunomodulatory compound through inhibition of lymphocytes proliferation and inducing their apoptosis [20]. The most abundant component of S. amplexicaulis oil (11.34%), caryophyllene oxide, was not present in S. juricis oil. This compound was recently reported as strong analgesic and anti-inflammatory as well as cytotoxic agent (21, 22). Germacrene D, identified only in S. amplexicaulis oil (7.79%), was previously described (23) to be very important odor agent in plant-insect interactions, while β-bourbonene showed antifeedant activity (24). Moreover, in the oil samples investigated in this study, compounds such as thujone and camphor were not present although such compounds that could provoke convulsions in central nervous system are reported for some Salvia species (25).

CONCLUSIONS

The study showed that oxygenated sesquiterpenes were the most abundant components in the essential oils of endemic S. juricis and S. amplexicaulis aerial parts collected from natural habitats in Macedonia. The chemical compositions of the investigated oils were qualitatively different regardless that samples were taken in close collection sites and during the same season. Therefore, it could be concluded that the differences are attributed to the chemotaxonomic identity of species rather than to the ecological conditions.

ACKNOWLEDGEMENTS

Authors are grateful to the Ministry of Education, Science and Technological Development of Serbia for financial support (Projects No. 173029 and 46013).

REFERENCES


ANTIOXIDANTS

Antioxidant activity and phenolic contents Pomegranate vinegar

KEYWORDS: Pomegranate vinegar, ORAC, TEAC, antioxidant, phenolic substances, ellagic acid.

Abstract Pomegranate is well known for its attributed health beneficial properties. The aim of this study was to determine the effects of ethanol and acetic acid fermentations on total antioxidant activities and phenolic substances of pomegranate juice. Juices of two types of pomegranates and their 1:1 mixture with pomegranate pomace (25%) were used to make pomegranate wine and vinegar. Total solids, pH, total soluble solids, total sugar, total phenolic substances, ORAC and TEAC assays, and phenolic substances analyses were carried out. Total phenolic content of Hicaznar and Beynar pomegranate juices were 2471.77 mg/L GAE and 1765.11 mg/L GAE, respectively. ORAC values of pomegranate vinegar samples were between 15.93-28.58 µmol/mL. Concentration of ellagic acid was the highest in pomegranate juice in all samples.

INTRODUCTION

Plant-based foods have a wide range of bioactive components that can provide significant beneficial health effects when consumed by humans. Fruits are rich in bioactive phenolic compounds such as flavonoids, phenolic acids, stilbenes, coumarins, and tannins (1). It was reported that a diet rich in flavonoids is associated with a reduction in the risk of hypertension, coronary heart disease and certain types of cancer (2). Pomegranate (Punica granatum) has high antioxidant activity due mainly to its inherent anthocyanins such as delphinidin, cyanidin and pelargonidone, and ellagittannins such as ellagic acid, punicalagin and punicaline (3,4). In the last decade, health benefits of pomegranate such as reducing the risk of cardiovascular diseases and certain types of cancer, improving the insulin sensitivity and preventing arthritis were reported (4,5). Lansky and Newman (4) stated that there are over 1000 cultivars of Punica granatum, mainly grown in the Mediterranean region, China, India and California (USA).

Fruit juices, wines and vinegars are good dietary sources of antioxidants (6,7). Vinegar has positive effects on health (8). Vinegar has effect which reduce of blood pressure (9), blood glucose response, and serum insulin (10). In addition, a potential antitumor effect (11), affirmative effects on blood lipid levels, liver functions and steatosis, and body weight increase (8) of apple cider vinegars has been expressed. Vinegar is obtained from agricultural raw materials containing starch or sugars by a double-fermentation process: the first one is ethanal fermentation and the second is acetic acid fermentation (12). In general, methods of manufacturing vinegar can be divided into two kinds: slow or traditional methods in which the culture of acetic acid bacteria is placed on the surface of a wood barrel, and quick or industrial processes involving submerged culture where the oxygenation should be increased so that the process is achieved at faster rates of acetification (13).

Previous studies have determined that wines and wine vinegars have significant antioxidant activity (8, 14-15). It was reported that pomegranate juice and pomegranate wine had significant protective effects toward low-density lipoprotein oxidation (16). Punica granatum L.cv. Hicaznar is a variety of pomegranate with a reddish peel and reddish arils and is the major pomegranate cultivar grown in Turkey; Punica granatum L.cv. Beynar variety has a pale red-whitish color arils and is another economically important cultivar in Turkey. In terms of flavor, Punica granatum L.cv. Hicaznar is more sour than Punica granatum L.cv. Beynar.

The aim of this study was to determine the changes in total antioxidant properties and phenolic substances as pomegranate juice is converted into pomegranate wine and pomegranate vinegar.

MATERIAL AND METHODS

Ethanol and acetic acid fermentations of pomegranate juice Punica granatum L.cv. Hicaznar and Punica granatum L.cv. Beynar were harvested from Korkuteli, Antalya, Turkey. Pomegranates were immediately transported to the Department of Food Engineering, Suleyman Demirel
University, Isparta, Turkey. Sample codes and sample descriptions were given in Table 1. The flow scheme of the process used to collect pomegranate juice is presented in Figure 1.

**Proximate analysis**

Collected pomegranate juice was examined as follows: pH was measured using a pH meter (WTW, Inolab, USA), total solids (%) was determined gravimetrically, and total soluble solid (°Brix) content of the samples was measured using Abbe refractometer (Bellingham Stanley Limit 60/70 Refractometer, England) (17). Total sugar was determined according to the Luff-Schrool method in the pomegranate juice samples (17).

**Total phenolic content**

Total phenolic contents of the pomegranate juice, wine and vinegar samples were determined spectrophotometrically according to the Folin-Ciocalteau method (18). The absorbance was determined at 760 nm using a spectrophotometer (Shimadzu Scientific Instruments, Inc., Tokyo, Japan).

**Oxygen Radical Absorbance Capacity (ORAC) assay**

ORAC assay was carried out according to the hydrophilic ORAC-Florescein method (6). The method kinetically measures the total antioxidant capacity of the samples using 2,2'-Azobis (2-aminopropane) dihydrochloride (AAAPH) damage to fluorescent compound. The Trolox® was used as a standard and ORAC values were kinetically calculated according to Cao and Prior (19) using KC4™ Data Reduction Software (BioTek Instruments, Winooski, VT).

**2,2’-azinobis (3-ethylbenzthiazoline)-6-sulfonic acid (ABTS) assay**

The 2,2’-azinobis (3-ethylbenzthiazolin-6-sulfonic acid) diammonium salt radical (ABTS) inhibition against Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was spectrophotometrically measured to determine Trolox Equivalent Antioxidant Capacity (20).

**Quantification of phenolics**

Gallic acid, catechin, epicatechin, chlorogenic acid, syringic acid and elagic acid in pomegranate juice, pomegranate wine and pomegranate vinegar samples were quantified by High Performance Liquid Chromatography (Shimadzu, Kyoto, Japan) equipped with an autosampler (SL-10AD vp), a reversed-phase column (Agilent Eclipse XDB-C18, 250x4.6 mm i.d., 5μm) and a diode array detector (lmax=278) (HPLC-DAD). The data were integrated and analyzed using the Data expressed as mean ± standard error.

\[ a,b \]

Values with the same lower-case letters in the same column do not differ significantly (P>0.05).

**Table 1. Sample descriptions and chemical analysis of pomegranate juice, wine and vinegar samples.**

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>Sample Description</th>
<th>pH</th>
<th>TSS (°Brix)</th>
<th>Total Solid (%)</th>
<th>Total Sugar (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPJ</td>
<td>Pomegranate juice from Punica granatum L. cv. Hicazan and Punica granatum L. cv. Beynr</td>
<td>3.14±0.14</td>
<td>17.76±0.4</td>
<td>16.10±1.4</td>
<td>169±54</td>
</tr>
<tr>
<td>RPW</td>
<td>Pomegranate wine made from Punica granatum L. cv. Hicazan and Punica granatum L. cv. Beynr</td>
<td>3.15±0.14</td>
<td>7.05±0.1</td>
<td>3.19±0.3</td>
<td>-</td>
</tr>
<tr>
<td>RPV</td>
<td>Pomegranate vinegar made from Punica granatum L. cv. Hicazan and Punica granatum L. cv. Beynr</td>
<td>2.87±0.1</td>
<td>7.15±0.1</td>
<td>2.77±0.2</td>
<td>-</td>
</tr>
<tr>
<td>WPJ</td>
<td>Pomegranate juice from Punica granatum L. cv. Beynr</td>
<td>3.27±0.14</td>
<td>14.85±1.6</td>
<td>14.62±0.5</td>
<td>170±21</td>
</tr>
<tr>
<td>WPW</td>
<td>Pomegranate wine made from Punica granatum L. cv. Beynr</td>
<td>3.20±0.0</td>
<td>5.35±0.2</td>
<td>1.33±0.0</td>
<td>-</td>
</tr>
<tr>
<td>WPV</td>
<td>Pomegranate vinegar made from Punica granatum L. cv. Beynr</td>
<td>2.88±0.0</td>
<td>6.05±0.1</td>
<td>1.5±0.1</td>
<td>-</td>
</tr>
<tr>
<td>MIXJ</td>
<td>Pomegranate juice from equal mixture of two pomegranate cultivars</td>
<td>3.15±0.14</td>
<td>17.0±0.0</td>
<td>15.63±0.5</td>
<td>180±22</td>
</tr>
<tr>
<td>MIXW</td>
<td>Pomegranate wine made from mixture of cultivar juices without macerated</td>
<td>3.29±0.0</td>
<td>7.0±0.7</td>
<td>3.23±0.3</td>
<td>-</td>
</tr>
<tr>
<td>MIXV</td>
<td>Pomegranate vinegar made from wine using mixture of cultivars without macerated</td>
<td>2.96±0.0</td>
<td>6.8±0.3</td>
<td>2.65±0.1</td>
<td>-</td>
</tr>
<tr>
<td>MIXPW</td>
<td>Pomegranate wine made from mixture of cultivar juices (maceration applied with 25% pomegranate arils and pulp)</td>
<td>3.29±0.0</td>
<td>5.9±0.2</td>
<td>2.33±0.3</td>
<td>-</td>
</tr>
<tr>
<td>MIXPV</td>
<td>Pomegranate vinegar made from wine using mixture of cultivars (maceration applied with 25% pomegranate arils and pulp)</td>
<td>2.83±0.0</td>
<td>0.75±0.4</td>
<td>2.18±0.0</td>
<td>-</td>
</tr>
</tbody>
</table>

![Figure 1. Flow scheme of ethanol and acetic acid fermentations of pomegranate juice.](image-url)
Shimadzu Class-VP Chromatography Laboratory Automated Software system. Standard and sample chromatograms are presented in Figure 2 and 3.

**Statistical analysis**

Differences among experimental data were analysed by ANOVA statistical program. Differences among experimental data were analysed by using SPSS for Windows (version 17.0, SPSS Inc.). Vinegar production was repeated two times. The significance was established at P < 0.05.

**RESULTS AND DISCUSSION**

The pH values, total solids (TS) and total soluble solids (TSS) of pomegranate juice, wine and vinegar samples, and total sugar content of pomegranate samples are presented in Table 1. The pH values of pomegranate juice obtained from Hicaznar and Beynar varieties of fruit were 3.14 and 3.27, respectively (P>0.05). The pH values of wine samples made from Hicaznar, Beynar, and their mixture with pulp were similar (P>0.05). Vinegar samples had the lowest pH values ranging from 2.87 to 2.96 due to high content of acetic acid; the effect of acetic acid fermentation on pH was significant (*P<0.05). The content of total soluble solids (TSS) is a significant parameter for wine production: contents of TSS for Hicaznar and Beynar juices were 17.75 Brix and 14.85 Brix, respectively (Table 1). Kumoro et al. (21) stated that yeast and initial sugar concentrations affected the production of wine. Higher yeast and initial sugar concentrations were found to inhibit the growth of the yeast cell.

Total sugar contents of Hicaznar and Beynar pomegranate juices were 169.64 g/L and 179.04 g/L, respectively. Contents of TSS and total sugar in both pomegranate juices were suitable for wine production. Ordoudi (22) stated that the content in total sugars of commercial pomegranate varieties (171 g/L) was higher than typical ones declared for fresh apple (100 g/L), mango (36 g/L) that are also used for vinegar production. Regardless of the pomegranate cultivar, wine and vinegar samples had a tendency to have lower TSS and TS than that of pomegranate juice samples (*P<0.05) due to the ethanol formation from sugar during alcoholic fermentation and removal of sedimental ingredients during wine making.

Total phenolic contents and TEAC (ABTS-) /ORAC values of pomegranate juices, wine and vinegar are presented in Figure 4 and Figure 5, respectively. TEAC and ORAC values of pomegranate juices in Hicaznar and Beynar pomegranate juices had 2471.77 mg GAE/L and 1765.11 mg GAE/L total phenolic substances.

**Table 2. Phenolic substances of samples.**
ORAC values RPV and WPV of pomegranate vinegar samples from 6.0 μmol TE/mL to 87.0 μmol of TE/mL. The two pomegranate varieties displayed significant differences in total antioxidant activities. Hicaznar has more reddish pigments while Beynar has pale red-whitish color. The presence of more color pigments in Hicaznar might be the reason for the high total antioxidant activities in Hicaznar and mixture samples. In a study conducted by Mousavinejad et al. [23] total phenolic substances and TEAC results of six pomegranate juice samples were reported and were in accordance with our results. Mixture of both variety juices had similar results with Hicaznar juice (P>0.05). However, the ORAC values of RPJ and MIXJ were significantly higher than the RPW and MIXPW, respectively; it should be noted that contents of ellagic acid were also significantly higher in RPJ and MIXJ samples than the contents of ellagic acid in pomegranate wine and vinegar samples (Table 2). Jin-wei [24] stated that there may not be a correlation between total phenolic contents and total antioxidant activities since the potential antioxidant activity was not solely from the phenolic contents for different fruits. Lee and Renaker [25] stated that there was a significant variation in ORAC values of wines; for example, ORAC values of Cabernet Sauvignon wine varied considerably from 6.0 μmol TE/mL to 87.0 μmol of TE/mL.

ORAC values RPV and WPV of pomegranate vinegar samples were 28.58 μmol/mL and 15.93 μmol/mL (P<0.05). The TEAC and ORAC values of MIXPV were significantly higher than that of WPV (P>0.05); the application of maceration resulted in increase of total antioxidant activity maybe due to the extraction of color substances from the reddish arids of Hicaznar pomegranate. All pomegranate vinegars had significant contents of total phenolic substances and total antioxidant activities in this study (P<0.05); actually these results were higher than the values reported in the literature for vinegars made from different raw materials. The grape wine vinegar exhibited ORAC values between 4.5 μmol TE/mL and 11.5 μmol of TE/mL [6]. Budak and Seydim [15] reported that total antioxidant activity values of traditional and industrial wine vinegars were 13.50 mmol/L and 10.37 mmol/L TEAC, respectively. The beneficial health effects of fruit vinegars may in part be related to the process-induced changes in their phenolics and generation of new antioxidative phenolics during fermentation [7]. Ellagic acid, gallic acid, catechin, epicatechin, chlorogenic acid, syringic acid, and p-coumaric acid were quantified in Hicaz and Beynar pomegranate juice, wine and vinegar samples (Table 2). Caffeic acid and ferulic acid were not detected in pomegranate juice, wine and vinegar samples. Ellagic acid is known as a significant bioactive component [26]. Pomegranate juices (WPJ, RPJ, MIXJ) had the highest content of ellagic acid in all samples, between 20.45 mg/L and 42.65 mg/L, respectively. Ellagic acid has also been identified and quantified in many fruits and fruit juices [27,28]. For example, Amakura et al. [27] reported ellagic acid contents of blackberry, strawberry, raspberry, bayberry, pomegranate, pineapple, apple, grape, prune juices. In our study, both ethanol and acetic acid fermentations of pomegranate juice resulted in significant destruction of ellagic acid (P<0.05).

Amakura et al. [27] stated that the content of ellagic acid was 14.3 mg/g while gallic acid was not found in pomegranate juice. Gallic acid was 0.00 mg/L in RPJ; 53.2 mg/L in WPJ; 58.35 mg/L in MIXJ. The significant differences observed between the contents of gallic acid in RPJ and WPJ (P<0.05) may be due to cultivar variability, environmental characteristics, period of harvesting or fruit maturity [26]. Phenolics range from simple, low molecular weight, single aromatic-ring compounds to the large and complex tannins and derived polyphenols. They are usually conjugated to sugars and organic acids [1]. Gallic acid is the most common phenolic acid and occurs widely as complex sugar esters in gallotannins such as 2-O-digalloyltetra-O-galloyl-glucose [28]. However, ethanol fermentation of RPJ increased the amount of gallic acid in RPW. Gallic acid has been liberated following to ethanol fermentation with sugar consumption by S. cerevisiae [15]. Pomegranate vinegar samples also contained significant amounts of gallic acid.

Contents of epicatechin were similar in two varieties of pomegranate juices (P>0.05). It gradually decreased in WPW, WPV and MIXPW, MIXPV. Some phenolic substances may have been lost during resettling and racking during vinegar production.

Variety and also acetic acid fermentation become important in the contents of catechin and chlorogenic acids since they were only identified in RPV and MIXPV [29]. In particular, the o-quinones of (+)-catechin and (−)-epicatechin were seen to be much less stable than those of chlorogenic acid [30].
CONCLUSION

Different varieties of pomegranate juices had different antioxidant properties and different profiles of phenolic substances. The content of bioactive ellagic acid is very high in both varieties of pomegranate juices. Ethanol and acetic acid fermentations resulted in changes in profiles of phenolic substances such as the destruction of ellagic acid. However, total antioxidant activities tended to increase in pomegranate wine samples due to liberation of other phenolics. Pomegranate wine and vinegar could not only be unique products due to their sensory properties but also provide significant contribution to health.

ACKNOWLEDGEMENT

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REFERENCES

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