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Healthy and Adverse Effects of Plant-Derived Functional Metabolites: The Need of Revealing their Content and Bioactivity in a Complex Food Matrix

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In recent years, both food quality and its effect on human health have become a fundamental issue all over the world. As a consequence of this new and increased awareness, American, European, and Asian policymakers have strongly encouraged the research programs on food quality and safety thematic. Attempts to improve human health and to satisfy people's desire for healthcare without intake of pharmaceuticals, has led the food industry to focus attention on functional or nutraceutical food. For a long time, compounds with nutraceutical activity have been produced chemically, but the new demands for a sustainable life have gradually led the food industry to move towards natural compounds, mainly those derived from plants. Many phytochemicals are known to promote good health, but, sometimes, undesirable effects are also reported. Furthermore, several products present on the market show few benefits and sometimes even the reverse - unhealthy effects; the evidence of efficacy is often unconvincing and epidemiological studies are necessary to prove the truth of their claims. Therefore, there is a need for reliable analytical control systems to measure the bioactivity, content, and quality of these additives in the complex food matrix. This review describes the most widespread nutraceutics and an analytical control of the same using recently developed biosensors which are promising candidates for routine control of functional foods.

Keywords Nutraceutics, plant metabolites, biosensors, antioxidant activity, safety, control

INTRODUCTION

Nutrition studies have provided unambiguous evidence that a number of human health conditions such as chronic coronary thrombosis, hypertension, diabetes, osteoporosis, cancer, old age, and lifestyle-related diseases are associated with the diet. Some human health disorders are often genetic, but there is a definite interplay of disorders/disease with contributions arising from consumption of certain, commonly used foods (Meydani, 2002; Desiere, 2004; Rist et al., 2006). In fact, in some cases, beneficial as well as harmful effects that have nothing to do with genetic predisposition have been identified in humans. For instance, diseases classified as polygenic in nature such as epithelial cancers, diabetes, and heart disease seem to be reduced by the intake of dietary antioxidants, vitamins and other phytonutrients. A gluten-free diet reverses the adverse health effects in celiac-disorder (Pizzuti et al., 2004; Niewinski, 2008); diminishing intake of galactose helps in the control of galactosemia (Boonyawat et al., 2005). Wide dissemination of such information has greatly helped to raise public awareness towards the consumption of food promoting good health and containing active ingredients to combat nutritional and health disorders (Mattoo et al., 2009). Prevention and therapy by means of individualized diets and supplements seem conceivable but their content and dosage to ensure minimal side effects need to be evaluated on a scientific basis through nutrition trials on humans.

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The amount of intrinsically beneficial nutraceutics in fruit, vegetables, whole grain cereals, and other foods, normally prescribed by physicians and nutritionists, is not always in accordance with the recommended daily allowance (RDA). Accurate information is also required about the RDA for effective phytonutrients, to name a few, vitamin C, carotenoids such as lycopene, carotenes and lutein, phenolics, flavonoids, minerals, etc., in order to determine biotechnological strategies for manipulating their contents in vegetables and fruits. Nutritional therapies are currently having limited success in establishing an appropriate control in preventing/and or stopping ongoing organ damage and in improving the survival rate, besides being time consuming and highly expensive.

To develop the concept of nutritionally functional food it is necessary to identify the biologically active molecules, to understand the mechanisms of prevention and protection, and to demonstrate the efficacy of these molecules by means of a large scale trial on humans. On the other hand, the identification of possible harmful factors like allergens and the determination of the bioavailability of an individual nutrient, whether provided as a food supplement or as enriched food is determined. Several studies highlight the fact that often the antioxidant capacity of fruits varies significantly according to the genotypes, species, and stage of growth, which can affect their nutritive value (Stadtman and Levine, 2003; Tripathi and Kamat, 2007). A typical example, in this context, is the discovery that pomegranate juice shows higher bioactivity than the individual polyphenols extracted from the fruit itself (Seeram et al., 2005).

PHYTOCHEMICALS AND ASSOCIATED PROBLEMS

Plants produce bioactive compounds which are classified into primary and secondary metabolites. Primary metabolites like carbohydrates, lipids, and amino acids, are necessary for the growth and basic metabolism in all plants, while secondary metabolites are not essential, but they may play crucial roles in plant wellbeing by interacting with the ecosystems. Compared to the main and most abundant molecules found in plants, the secondary metabolites defined by their low abundance, often form less than 1-5% of the dry weight. Some secondary metabolites are exploited for promoting public health as they provide health benefits in addition to basic nutrition (Diplock, 1999; Krzyzanowska et al., 2009; Wahle et al., 2009).

These molecules are often produced under plant stress and largely contribute to plant fitness. They have been described as being antibiotic, antifungal, and antiviral, and therefore are able to protect plants from pathogens, and also to inhibit germination of phytotoxics in other plants. Besides, they constitute important UV absorbing compounds which prevent DNA and photosynthetic apparatus damage (Mattoo, 1999; Wink, 2003; Hartmann, 2007; Wallace, 2007). They can also influence animal performance or behavior, for example, insects (anti-feeding properties) or even cattle (whose forage grass contains estrogenic properties which interact with fertility) (Iason, 2007; Harris et al., 2007; Hignett, 2007). Due to their important biological activities, plant secondary metabolites have been used for centuries in traditional medicine. Nowadays, they find applications in cosmetics, fine chemicals, and more recently in nutraceutics or functional food.

The production of plant secondary metabolites has, for a long time, been achieved through chemical synthesis, but their production is also possible through the field cultivation of medicinal plants. However, plants originating from particular biotopes hardly ever succeed in growing outside their local ecosystems. It is often the case that common plants cannot withstand field cultivation due to their sensitivity to pathogens (Kelly and Vallejo, 2004; Abang et al., 2005; Talhinhas et al., 2005; Garrido et al., 2008). This behavior has led scientists and biotechnologists to consider plant cell, tissue, and organ cultures as alternative ways of producing the corresponding secondary metabolites. Plants react against these external attacks, such as pathogens, by means of a protection mechanism which leads to the production of secondary metabolites. Several other issues are associated with the use of nutraceutics and concern the dose, activity, and presence of contaminants. Phytochemicals, if in excess, can result in undesirable effects; for example, high doses of carotenoids have been associated with an increased risk of lung cancer in smokers (Satia et al., 2009) and in alcohol drinkers (Ratnasinghe et al., 2000). Moreover, several products present on the market have little or no effect due to incorrect preparation and storage. In the enriched metabolic extracts, the presence of undesirable compounds is often a reality, such as the case of pesticides and heavy metals, or other toxic natural chemicals; for instance, α-thujone, a natural monoterpene, is present as an active component in various herbal products such as the extracts of *Salvia officinalis* (Ozkan et al., 2009) and of *Artemisia absinthium* (Teixeira da Silva et al., 2005; Lopes-Lutza et al., 2008). In the scientific literature, beneficial effects are reported for these plant extracts which possess antioxidant, bactericidal, and antimicrobial activities but α-thujone is also known for its psychoactive effects and its toxicity has become an important issue in recent years (Deiml et al., 2004; Haji Mahdipour et al., 2008; Kharoubi et al., 2009).

The functional food industry has marketed foods enriched with bioactive compounds, but there are no universally accepted criteria for judging the efficacy of these compounds.

The main scepticism of consumers regarding functional foods resides in the veracity of health claims and in the poor and often inadequate control of their proclaimed properties. Legislation concerning this matter is progressing at an extremely low pace and currently only Japan, the U.K., the United States, and Scandinavian countries have managed to make noticeable progress. Recently, the maximum limits for these substances have been fixed in the Regulation (EC) No. 1924/2006 of the European Parliament and of the Council of 20 December 2006 on Nutrition and Health Claims made on Foods. Moreover, the labelling of functional foods is far from informative, providing scanty details about nutritional value, storage, and cooking

recipes. It is anticipated that technological advances in the food industry, in conjunction with extensive clinical trials and governmental control, will eventually guarantee the credibility of health claims and strengthen the belief of consumers in functional foods.

Therefore, due to the complexity of the food matrix there is a need for reliable analytical systems to measure the content, quality, activity, and safety of the final product. Classical methods, such as HPLC and GC/MS, although economically viable, often involve lengthy preparation procedures when dealing with the complex matrices of real foodstuffs. Biosensors are new and appealing analytical tools based on biological materials; they are promising means of coping with the complexity of functional food matrices. This review proposes new methods together with a process for determining whether there is reasonable evidence of efficacy and safety of nutraceutical products or functional food. Before describing it, the most interesting groups of compounds currently marketed by the functional food industry are overviewed. Thus, polyunsaturated fatty acids, carotenoids, phenolic compounds, alliin, allicin, glucosinolates, and capsacinoids, are briefly described in this section.

THE PHYTOCHEMICALS PRESENT ON THE CURRENT MARKET

Polyunsaturated Fatty Acids (PUFAs)

PUFAs are long-chain unsaturated carboxylic acids having more than one double bond. There are several different systems of nomenclature in use for fatty acids; some of them use the historical names or the most largely found in scientific literature; others distinguish fatty acids on the basis of their structural or chemical properties. Systematic names derive from the standard IUPAC Rules for the Nomenclature of Organic Chemistry which is very elaborate, but, technically clear and descriptive. The preferred nomenclature when dealing with PUFAs in the field of nutraceutics and functional metabolites is the so called '$n-x$' or 'ω-x' which originates from the physiological properties of these compounds. Following this convention, n or ω represents the terminal methyl carbon of PUFA chain while x is the carbon–carbon bond where the double bond is located.

Recent dietary data show a tendency towards the increased consumption of essential fatty acids –PUFAs having a carbon–carbon double bond in the n−3 or n−6 position as n–6 and n–3 fatty acid families show important biological functions for human metabolism (Yokoyama et al., 2007). These fats are considered essential nutrients since they cannot be produced by the body and need to be supplied by a diet. α-Linolenic acid, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) are $n-3$ fatty acids important in human nutrition. These three polyunsaturated acids have chains of 18, 20, or 22 carbon atoms, respectively; linolenic acid and arachidonic acid are $n-6$ fatty acids which are important in human nutrition. These compounds have carbon chains of 18 and 20 carbon atoms, respectively (Fig. 1).

Dark flesh fish such as herrings, mackerels, sardines and salmons, seafood, and fish oil supplements are rich sources of omega-3 PUFAs; α-linolenic acid is also present in leafy green vegetables, purslane, walnuts and perilla seed, pumpkin seed, flaxseed and rapeseed oils, and soybean and canola oils (Mangat, 2009; Rajaram et al., 2009; Craig, 2009).

The consumption of the omega-3 fatty acids EPA and DHA by adults is strongly correlated with reduced risk of cardiovascular disease (Lavie et al., 2009; Wang and Widlansky, 2009). They possess anti-inflammatory effects as well as being cardioprotective including anti-arrhythmic, anti-thrombotic, they lower the blood pressure, improve the endothelial function, and slow down the growth of atherosclerotic plaque; furthermore, they have antibiotic-like effects (Desbois et al., 2009). Higher omega-3 PUFAs concentrations cause higher membrane fluidity, which increases serotonin transport (Mazza et al., 2007). Linoleic acid is important in the biosynthesis of arachidonic acid which is the precursor for prostaglandins and other physiologically active molecules. The fatty acid composition determines the biophysical properties of neuronal membranes and affects neurotransmission.

Omega-3 fatty acids also play an essential role in the development and activity of the central nervous system, improve cognitive development and reference memory-related learning

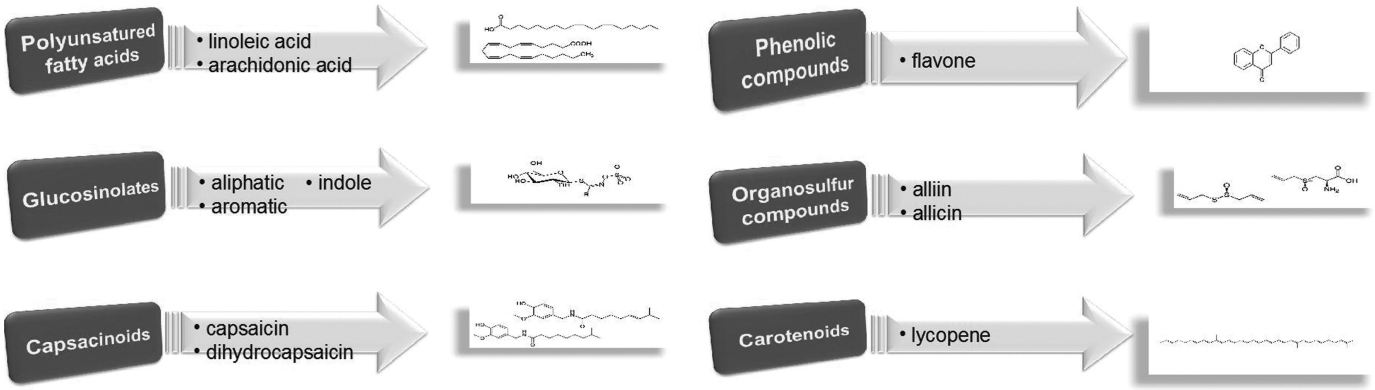

Figure 1 Main nutraceutic phytochemicals applied in functional food industry. (Color figure available online.)

and are involved in various neurodevelopments (Beltz et al., 2007; Mazza et al., 2007). Interestingly, DHA is an essential compound of newly born and young infants' diets because their organisms are unable to produce it at the rate sufficiently fast to keep up with the demands of the growing brain, hence an external source of PUFAs is necessary (Guesnet and Alessandri, 2010).

Nevertheless, medical research suggests that excessive levels of $n-6$ fatty acids relative to $n-3$ fatty acids may increase the probability of a number of diseases and/or depression (Okuyama et al., 2007; Dinan et al., 2009; Mimoun et al., 2009). Actually, modern diets typically have ratios of $n-6$ to $n-3$ in excess of 10 to 1, with some as high as 30 to 1. The optimal ratio is thought to be 4 to 1 or lower (Simopoulos, 2008; Russo, 2009).

Carotenoids

Carotenoids are red, orange, and yellow lipid-soluble pigments embedded in the membranes of chloroplasts and chromoplasts. One of the most important and well-known members of the carotenoid family is lycopene, depicted in Fig. 1, which is an intermediate for the biosynthesis of many carotenoids. The most abundant carotene is beta-carotene, and xanthophylls are oxidized derivatives of carotenes which include several compounds, that is, zeaxanthin, neoxanthin, violaxanthin, and α- and β-cryptoxanthin. Both carotenes and xanthophylls belong to the polyisoprenoids and contain 40 carbon atoms formed by the condensation of eight isoprene units. Carotenoids possess a long chain of conjugated double bonds whose linkage order is reversed in the central part of the molecule giving rise to a symmetrical molecule. This set of conjugated double bonds is responsible for the absorption of light in the visible region of the spectrum. Different levels of hydrogenation and the introduction of oxygen-containing functional groups in the left and right end chains create a large family of over 600 natural compounds (Nagini, 2008). Their effect on the human body is to protect the organism against the oxidative damage caused by an excess of highly aggressive chemical species.

Animals are incapable of synthesizing carotenoids and must obtain them through their diet. Lutein and zeaxanthin are the only carotenoids profusely present inside the human body in the macular region and the crystalline lens of the eye (Humphries and Khachik, 2003; Trevithick-Sutton et al., 2006). In plants, lutein and zeaxanthin accumulation levels are lower compared to carotenes. The function of these pigments is to improve the eyesight, quenching free radicals and in so doing act as antioxidants to protect the macula from oxidative damage. Lutein and zeaxanthin protect eyes against photo-induced damage by shielding against UV light and potentially harmful short-wave radiation. Significant correlations have been found between lutein and zeaxanthin concentrations in ocular tissues, serum, and plasma, and a possible reduction in the risk of macular degeneration (Kintzios et al., 2003).

However, findings from prospective cohort studies suggest that an inverse association between carotenoids and lung cancer can occur. Poor nutritional habits (i.e., absence of fruit and vegetable consumption) together with smoking have been associated with statistically significantly elevated risk of total lung cancer (Gallicchio et al., 2008; Satia et al., 2009). Adverse effects have also been reported in diabetic patients subjected to excessive beta-carotene food-intake (Mikkelsen et al., 2009); thus, the conclusion is that more clinical trials are needed.

Phenolic Compounds

The phenolic structure is present in simple, low molecular weight compounds with a single aromatic ring as well as in very large, complex, and multi-ringed species (Goldberg, 2003). Polyphenols include several thousand compounds, among them the flavonols, flavones, catechins, flavanones, anthocyanidins, and isoflavonoids (Le Marchand, 2002). Flavonoids derive from 2-phenyl-1,4-benzopyrone (commonly named flavone) whose molecular structure is presented in Fig. 1. In plants, flavonoids are responsible for various functions like pigmentation of leaves and fruits, attracting and repelling insects as well as protecting plants from herbivores (Iwashina, 2003; Koes et al., 2005). In the human body, most of the beneficial health effects are attributed to their antioxidant, free radical scavenging action as well as metal chelating ability. This is the reason why the capacity of flavonoids to inhibit low-density lipoprotein oxidation is thought to play an important role in prevention of cardiovascular diseases (Scalbert et al., 2005; Hooper et al., 2008). However, in recent years, following the achievement of the human genome sequencing, the exploitation of molecular biology tools revealed that specific antioxidant compounds modulate various cellular functions, acting not only as radical scavengers, but also as genes and enzyme expression regulators (Knasmüller et al., 2008). Several studies showed that flavonoids may act as antiproliferative and anticarcinogenic agents. They inhibit carcinogenesis, in both in vitro and in vivo experiments, by affecting the molecular events in the initiation, promotion and progression state of cancer. Moreover, some flavonoids are reported to have anti-inflammatory activity, which is related mainly to their ability to inhibit the production of inflammatory mediators such as prostaglandins, leukotrienes and nitric oxide (Lin and Weng, 2006).

Four different families of phenolic compounds, that is, isoflavones, stilbenes, lignans, and coumestans are considered as phytoestrogens or plant estrogens. All of them are similar in structure to estradiol and possess estrogenic activity due to the fact that they have an 'A' ring similar to that of estradiol as well as possessing two hydroxyl groups positioned at a correct distance one from the other that facilitates binding to the estrogen receptors (Eskin and Tamir, 2006; Martin et al., 2007). The estrogenic activity of these compounds was first discovered in the 1940s, when they were indicated as the cause of infertility of Australian sheep who grazed on clover (*Trifolium*

subterraneum) whose leaves contain high amounts of the isoflavons biochanin A and formononetin (Cornwell et al., 2004).

All the same, controversial effects of polyphenols have also been demonstrated. Resveratrol, which is a stilbene derivative, for example, can improve lipid profile and glucose level in the case of high-fat diets, but produces hepatic oxidative stress in standard-fed diets (Rocha et al., 2009). Epigallocatechin-3-gallate (EGCG), which is the main polyphenolic constituent in green tea, is known for its antioxidant power, but, in excess can promote the formation of radical species causing oxidative cell damage (Suh et al., 2009). In addition, the breakdown products of phenolic compounds can act as anti-nutritional factors in diet (Jahangir et al., 2009).

Alliin and Allicin

Alliin (cysteine sulfoxide) and allicin (cysteine thiosulfinate) (Fig. 1) are two important organosulfur compounds highly concentrated in garlic (*Allium sativum*). Allicin is formed by the action of the enzyme alliinase on alliin which is a derivative of the amino acid cysteine. Allicin is often considered the "active ingredient" in garlic, and has been the subject of numerous scientific studies. However, this molecule is unstable and is rapidly converted into mono-, di-, and trisulfides and other compounds, many of which have been reported to have medicinal effects on a variety of diseases that are, however, less efficient than allicin (Lawson and Hughes, 1992). These compounds are considered protective agents in cardiovascular diseases, because they significantly reduce serum cholesterol and triacylglycerol levels, and exercise a hypolipidemic, anti-hypertensive, anti-diabetic, and antithrombotic activity. Moreover, they exhibit antioxidant properties thereby preventing atherosclerosis (Sato and Miyata, 2000; Jakubowski, 2003; Corzo-Martínez et al., 2007) and are protective against many fungi, virus, and bacteria (Corzo-Martínez et al., 2007). Organo-sulfur compounds inhibit also tumour cell growth and cellular proliferation, influencing the DNA repair mechanisms that shows an antimutagenic activity (Jakubowski, 2003; Wu et al., 2004; Corzo-Martínez et al., 2007). Diallyl trisulfide and diallyl sulfide stimulate T cell proliferation and macrophage cytotoxicities on tumor cell lines as well as increasing the activity of detoxifying enzymes.

Glucosinolates

Glucosinolates are nitrogen and sulphur-containing secondary plant metabolites present in high concentration in all cruciferous plants. There are about 120 defined glucosinolates. All molecules share a common chemical structure consisting of a sulfonated moiety, a β-D-thioglucose group, and a variable side chain derived from one of eight amino acids (Fig. 1). Glucosinolates can be classified into three chemical groups depending on their amino acid precursor. Compounds derived from alanine, leucine, isoleucine, methionine, or valine are called aliphatic glucosinolates, those derived from phenylalanine or tyrosine are called aromatic glucosinolates, and those derived from tryptophan are called indole glucosinolates (Johnson, 2002; Halkier and Gershenzon, 2006). Most of the biological activities of glucosinolates can be attributed to the actions of their hydrolysis products, isothiocyanates or some indolic compounds, which reduce the risk of carcinomas of the lung, stomach, colon and rectum (Johnson, 2002; Eskin and Tamir, 2006). The isothiocyanate sulforaphane derivative of 4-methylsulfinylbutyl glucosinolate and other isothiocyanates may prevent tumor growth by blocking the cell cycle and promoting apoptosis. Moreover, it has been shown that glucosinolate singrin causes a reduction of precancerous lesions in a dimethylhydrazine induced colon cancer model of a rat (Halkier and Gershenzon, 2006). On the contrary, some negative effects are reported about glucosinolates mainly regarding their effect above on the thyroid gland in various animals since the ingestion of over doses causes an abnormal absorption of iodine by the gland, provoking hypertrophy and goiter (Das et al., 2000).

Capsaicinoids

Capsaicinoids belong to plant secondary metabolites that contain nitrogen and to the family of aromatic fatty amides (Fig. 1) produced by chilli peppers. The main capsaicinoid in chilli peppers is capsaicin which typically amounts to 69% of the capsacinoid mixture and the second most abundant species is dihydrocapsaicin which forms 22% of the mixture/content. Other capsacinoids such as nordihydrocapsaicin, homocapsaicin, nonivamide, and homodihydrocapsaicin are also present (Surh et al., 1995; 1996; Eskin and Tamir, 2006) and constitute the remaining part of the mixture. Capasaicin and the other capsaicinoids possess interesting pharmacological actions for humans. Capsaicin selectively affects the peripheral part of the sensory nervous system reducing pain. For this reason it is used in pharmacotherapy to treat rheumatoid arthritis, post-herpetic neuralgia, post-mastectomy pain syndrome, and diabetic neuropathy. It has been proven that topical cream containing 0.025% capsaicin significantly relieved the pain in patients with arthritis (Surh and Lee, 1995; 1996). Moreover, numerous studies have focused on the anticarcinogenic and antimutagenic properties of capsaicin (Surh and Lee, 1996). However, in spite of the beneficial effects of these metabolites on stomach cancer, in people consuming chilli, there is also a confirmed study that the high consumption of chilli pepper by rats promotes a higher risk for them of getting gastric cancer (Surh and Lee, 1996; Aherne and O'Brien, 2002).

CONTROL ANALYSES BY BIOSENSOR TECHNOLOGY

While, on the one hand, this knowledge on phytochemicals has led to a growing interest and attention towards targeted nutrition and the fast growth of functional and enriched food on the

market, on the other hand, only little emphasis has been placed on the analytical aspects. In this regard, specific, new technologies have recently been developed to examine nutraceutical components.

Biosensors are a novel and appealing alternative to the classic methods for the routine analysis of industrial products. A huge range of applications is targeted/has been targeted such as the agricultural sector, veterinary analysis, beverage industry, fermentation industry, waste water management, monitoring of environmental pollution, microbial contamination, clinical diagnosis, drug monitoring and analysis, mining, military and defence sectors, aerospace personnel safety and many others. A biosensor is a self-contained integrated device, which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) which is placed in direct spatial contact with a transduction element. Biosensors may be classified according to the specific biological activity mechanism or, alternatively, to the mode of physico-chemical signal transduction. The biological recognition element may be based on a chemical reaction, or on an equilibrium reaction with macromolecules that have been isolated, engineered or are present in their naive biological environment. In the latter cases, equilibrium is generally reached and there is no further, if any, net consumption of analyte(s) by the immobilized biocomplex agent incorporated in the sensor. Biosensors may be further classified according to the analytes or reactions that they monitor such as direct monitoring of analyte concentration or of reactions producing or consuming such analytes, respectively. Alternatively, an indirect monitoring of an inhibitor or activator of the biological recognition element (biochemical receptor) may sometimes be achieved (Thévenot et al., 2001).

The biological element can be a system containing enzyme(s), plant or animal tissues, microorganisms, organelles, cell receptors, antibodies, nucleic acids, etc. The transduction element is a material possessing electrochemical, optical or piezoelectric properties (see Fig. 2).

According to the recent scientific literature the biosensors dominating the food sector of the market in the last three years are related to electrochemical transduction systems. Despite the enormous diversity of research involving biosensors, there have been few applications in nutraceutical analysis. Since 1998 slightly more than 100 articles have been published containing the word "biosensors" and "food" and in 2008, 17 studies appeared. Biosensors able to evaluate the activity and the content of nutraceutical metabolites or detect the presence of contaminants both in vitro cultures and in complex food matrices have been developed. In the following section attention is focused on those biosensors best established in the field of phytochemicals.

Revealing Phytochemical Antioxidant Activity

When assessing phytochemical compounds, the main interest is in quantifying their activity, evaluating how successful they are in promoting good health, and preventing diseases. The

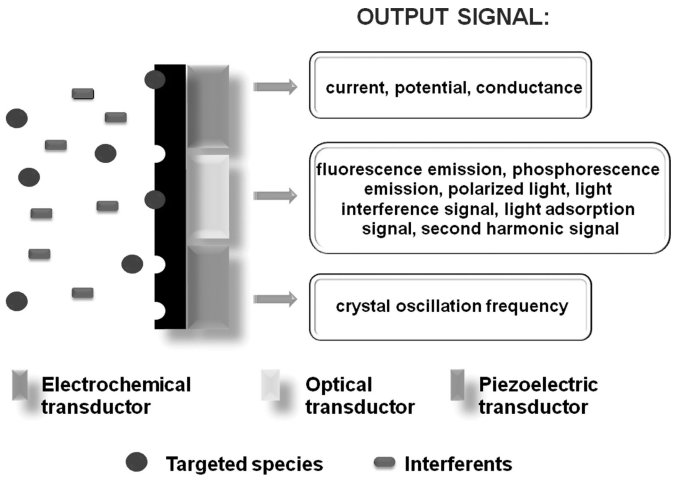

Figure 2 General scheme for biosensors: a biological sensing element is in contact with a physicochemical transductor. When the biomolecule recognizes the target analyte, a reaction occurs producing a signal converted and detected by the transductor. (Color figure available online.)

protective mechanisms of plant metabolites involve a variety of physiological functions acting as direct or indirect antioxidants, regulating enzymes, and controlling apoptosis and the cell cycle. The literature of biosensor research mainly refers to the ability of phytochemicals to act as shields against free radicals. Some radicals are produced in the human body as natural by-products of the normal metabolism of oxygen and are known as "reactive oxygen species." In organic chemistry free radicals frequently originate from breaking the bond between atoms that have similar electron negativity such as peroxide or oxygen-nitrogen. When these molecules or pro-radical reactive species are present in excess in the human body, oxidative damage can occur at the molecular level (Somogyi et al., 2007). Biosensors designed to detect antioxidant activity in phytochemical compounds are commonly based on cytochrome c, superoxide dismutase, and DNA.

Cytochrome c-based biosensors are electrochemical devices using this redox protein as a bio-recognition element and the superoxide radical anion ($O_2^{\bullet -}$) as a reactive oxygen species to reveal the scavenging capacity of antioxidant compounds. Cytochromes c (Cyt c) are mobile electron-transfer proteins embedded in the inner membrane of mitochondria that play an important role in the biological respiratory chain as an electron transporter from cytochrome c reductase to cytochrome oxidase. They are ubiquitous mobile proteins containing the haem prosthetic group in which iron is found in the reduced Fe(II) or oxidized Fe(III) form. The iron atom of the haem group of Cyt c is the actual iron carrier (Wu and Hu, 2007). In the biosensor, Cyt c, immobilized on the electrode surface, is reduced in the presence of superoxide ion and immediately regenerated by the polarized electrode, producing a current whose intensity is directly proportional to the radical concentration (Cortina-Puig et al., 2009). So, the addition of radical scavenging compounds decreases the signal intensity, demonstrating the capability of the examined species to act as an antioxidant (Dronov et al.,

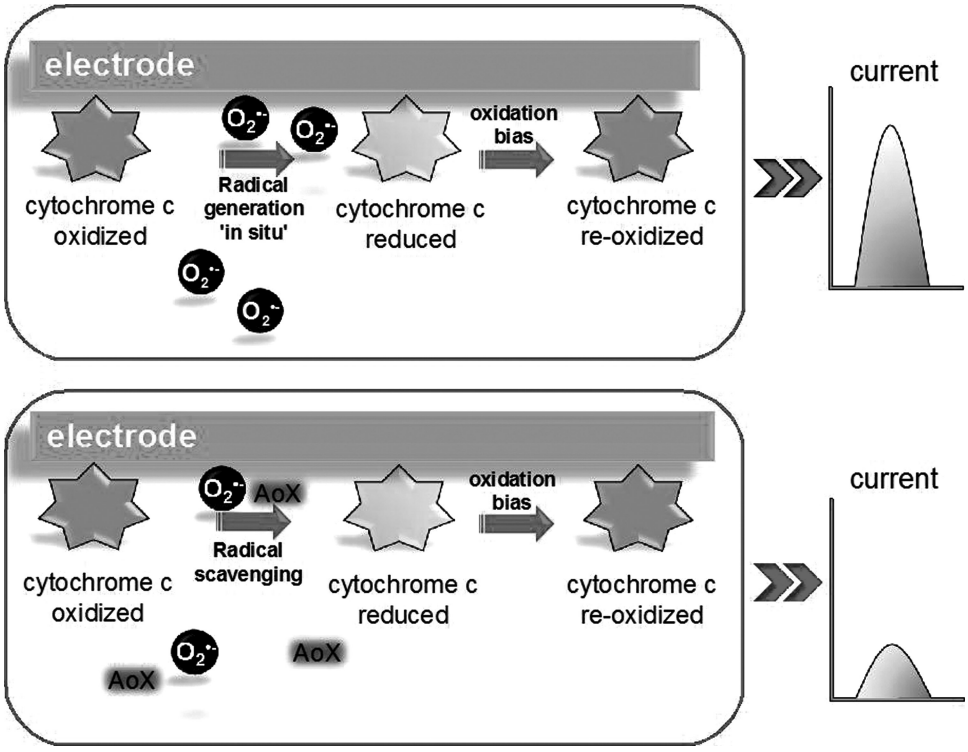

Figure 3 Cytochrome c-based biosensor for the measurement of antioxidant capacity. The in situ generated superoxide radical anion ($O2^{\bullet -}$) is oxidised by the biomolecule while the reduced Cytochrome c is suddenly reoxidised onto the electrode surface. The produced signal is proportional to the radical concentration, but if an antioxidant species is added, a radical scavenging occurs causing a lowering in the detected signal. (Color figure available online.)

2007; Prieto-Simón et al., 2008). The reaction which occurs is represented in Fig. 3.

The performance of these types of biosensors greatly improves if an enzyme capable of generating radicals directly in situ is co-immobilized on the device.

This is the case when Cyt of c is co-immobilized with xanthine oxidase (XOD). The latter generates superoxide following the hypoxanthine (HX) oxidation ($HX + H_2O + O_2 \rightarrow$ uric acid $+ 2\ H^+ + O_2^{\bullet -}$) (Mello and Kubota 2007). However, the spontaneous dismutation of $O_2^{\bullet -}$ in H_2O_2 in the presence of protons ($2\ O_2^{\bullet -} + 2\ H^+ \rightarrow H_2O_2 + O_2$) cannot be avoided. The hydrogen peroxide production can cause interference with biosensor measurements, since the interaction of H_2O_2 with reduced Cyt c leads to a protein re-oxidation; for this reason, recent studies have developed Cyt c-based systems equipped with Cyt c/XOD, but also with enzyme catalase, which catalyzes the decomposition reaction of hydrogen peroxide into water and oxygen (Wu et al., 2007).

Similarly to the cytochrome c-based biosensors, superoxide dismutase biosensors use the radical anion $O_2^{\bullet -}$, produced by the action of the xanthine/xanthine oxidase biochemical system as reactive oxygen species to be scavenged by a compound with presumed antioxidant capacity. However, there is a fundamental difference in the mechanism of action of these two types of biosensors; in the former case the immobilized cytochrome c directly reacts with $O_2^{\bullet -}$ whereas in the latter case the superoxide dismutase-based biomediator catalyzes the dismutation of the $O_2^{\bullet -}$ producing molecular oxygen and hydrogen peroxide. Both these species can be reduced on the electrode surface under a properly applied bias; generally hydrogen peroxide reduction is preferred. The current produced is proportional to the concentration of the radicals, which decreases when antioxidant species are added, causing a lowering in the current signal. The current signal variation allows the antioxidant capacity to be quantified. The mode of action of superoxide dismutase-based biosensors is dual: event (a) pertains to those biosensors in which oxygen is reduced, while event (b) pertains to the majority of superoxide dismutase biosensors in which H_2O_2 is reduced (see Fig. 4).

The development of different superoxide dismutase-based biosensors for assessing the antioxidant capacity of several compounds was reported (Campanella et al., 2005; Bonanni et al., 2007). Most of this work is based on the immobilization of superoxide dismutase in a α-carrageenan gel and the amperometric detection of H_2O_2. This biosensor has been used to evaluate fresh aromatic herbs, olives and fresh fruit, bulbs and vegetables, plant products sold by herbalists or chemists, teas, phytotherapeutic diet integrators, and drugs containing β-carotene (Campanella et al., 2000).

DNA-based biosensors are obtained by immobilizing DNA strands on the surface of an electrode. To test the activity of the biosensors, radicals are produced by oxidizing a transition metal cation such as iron Fe(II), copper Cu(I) or chromium Cr(II) with hydrogen peroxide. The resulting hydroxyl radical ($^{\bullet}OH$) reacts directly with DNA destroying the guanine bases of the double helix. The antioxidant activity is determined by studying the variation of the oxidation peak of guanines in square wave

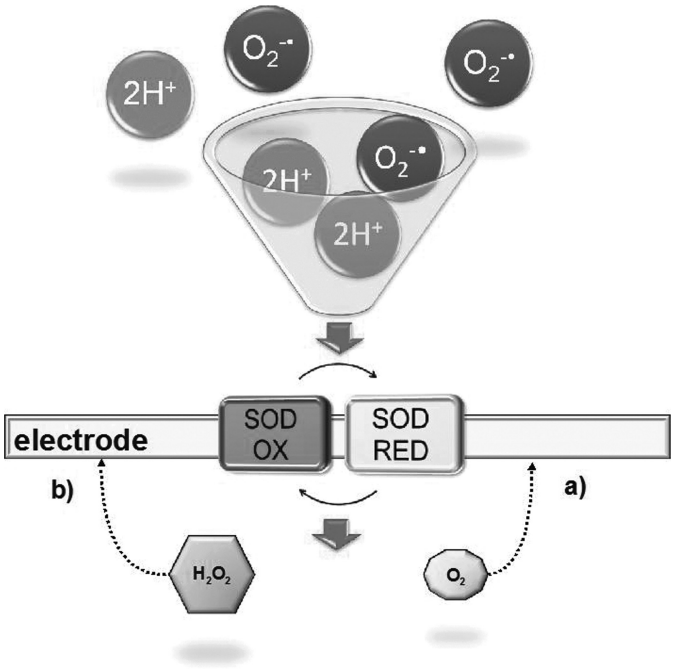

Figure 4 Superoxide dismutase- based biosensor for the measurement of antioxidant capacity. The superoxide radical anion ($O2^{\bullet -}$) is generated in situ and converted by the protein to molecular oxygen and hydrogen peroxide. One of these products (generally hydrogen peroxide) is reduced onto the electrode surface producing a current proportional to the radical concentration (a) or (b). When an antioxidant is added, it reacts with the radical and a lower signal is detected. (Color figure available online.)

voltammetry. The working mechanism of DNA-based biosensors is schematically reported in Fig. 5. The use of superoxide and DNA-based biosensors were reported to detect the antioxidant capacity of polyphenolic compounds in wines, various teas, and herbal products such as camomile, dog rose, and ginseng (Campanella et al., 2004). Mello et al., 2003; 2006 used these biosensors based on $^{\bullet}$OH radical generation to analyze the presence of polyphenols in plant extracts from *Baccharis genstelloides*, *Peumus boldus*, *Foeniculum vulgare*, *Cymbopogom citrates*, *Camellia sinensis*, and *Mentha piperita.* Other articles by Cortina-Puig et al., 2009 and Litescu and Eremia, 2009 report DNA-based antioxidant biosensors using differently selected radical species such as $O_2^{\bullet -}$ and nitric oxide radical ($NO^{\bullet}$), adding further information and results to the numerous configurations which had already appeared in the literature. Equally, these biosensors are applicable in developing possible models for in vitro assessment of antioxidant or the radical-scavenger capacity of polyphenols, flavonoids, isoflavones, anthocyanines, etc.

Revealing Content of Functional Metabolites

By far the greatest number of biosensors developed for the monitoring of nutraceutical components aim at determining the concentration of polyphenolic compounds, particularly flavonoids. The polyphenol content is evaluated using different types of biomediators such as tyrosinase, laccase, horseradish peroxidase (HRP) and HRP/DNA mixtures. Tyrosinase and laccase belong to the protein class of phenol oxidases which catalyze the oxidation of phenolic compounds to the correspondent quinones by molecular oxygen. The quinone species formed in the solution are electrochemically further reduced producing a current whose intensity is directly correlated to the phenol concentration. HRP and HRP/DNA-based electrodes exploit the peroxidase activity of the HRP enzyme which belongs to the protein class of peroxidases. These latter enzymes catalyze the oxidation of phenolic compounds by hydrogen peroxide. Also, in this case, quinone species are formed that can be further reduced on the electrode surface with a current proportional to the phenol concentration and resulting in an amperometric device (Mello et al., 2003).

A few articles also report on the concentration of glucosinolates (Wu et al., 2005) in which the evaluation has been carried out using biosensors made of myrosinase and glucose oxidase enzymes, both for electrochemical and optical biosensors. Myrosinase and glucose oxidase are co-immobilized on a Clark-type oxygen electrode. In the electrochemical device, myrosinase catalyzes the hydrolysis of glucosinolates to glucose. Subsequently, the glucose is oxidized by glucose oxidase causing the consumption of oxygen. The oxygen level is monitored and related to the glucosinolates concentration (Wu et al., 2005). The optical device utilizes analogous chemical reactions but the glucose is determined by means of a glucose oxidase-immobilized eggshell membrane and an oxygen-sensitive optode membrane (Choi et al., 2005).

A class of biosensors capable of detecting alliin has also been developed. They are based on the activity of the immobilized enzyme allinase which catalyzes the oxidation of alliin to allicin producing ammonia. The biosensor detects just this compound using an ammonia gas electrode or a pH sensitive electrode (Keusgen et al., 2003; Turek et al., 2008). Regarding the determination of capsacinoids, multiwalled carbon nanotube screen-printed electrodes have been used to assess the capsaicin content in chilli pepper, hot sauce, and other related foodstuffs. The quantification was determined by means of an adsorptive stripping voltammetry technique (Kachoosangi et al., 2008).

Up to now, the quantification of fatty acids and carotenoids by applying biosensor technology cannot boast of outstanding success; conventionally, these metabolites are still detected using classical methods. However, due to their importance and widespread use, preliminary attempts have been made to quantify their antioxidant activity (Tibuzzi et al., 2007).

Revealing Safety and Quality of Nutraceutics

The absence of microbial and chemical contaminations is an important issue when producing stuff for human nutrition. Phytochemicals are isolated both from plants and in vitro cultures developed specifically for their mass production.

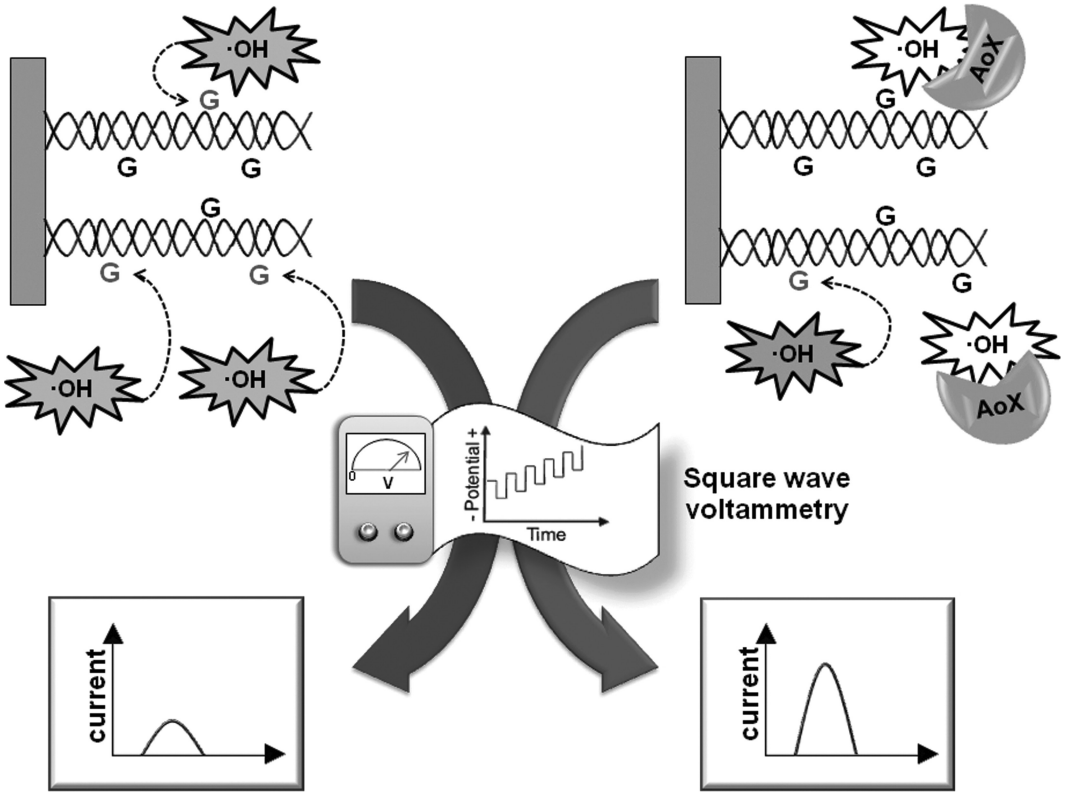

Figure 5 DNA-based biosensor for the measurement of antioxidant capacity by means of square wave voltammetry: left) DNA strands are immobilized onto the surface of a Screen-Printed Electrode (SPE): in the absence of antioxidant species (AoX) a low current signal, induced by the oxidative damage of guanine bases in DNA helix, is detected by the electrode; right) in the presence of AoX, free radical are scavenged and the guanine bases preserved, thus a higher current signal is registered. (Color figure available online.)

In vitro culture techniques are a useful tool to increase the production and marketing of plant species because they allow a rapid clonal propagation of plants selected for their active principles and overcome many problems linked to conventional agriculture such as variations in crop quality due to environmental factors: drought, flooding, and other abiotic stresses, and/or biotic stresses such as pathogen-induced diseases. Moreover, in vitro cultivation supplies continuously plant material overcoming the problem of the alternation of bioactive compound production, resulting from variable climatic conditions caused by the seasons and geographic location. Among the various factors affecting culture growth and plant secondary metabolite production, the agitation systems and sterilization conditions play an important role: non-optimal/incorrect sterilization conditions, adopted during the production of nutraceutics can lead to the presence of pests or contaminants which negatively influence the whole process. For this reason the monitoring of cell cultures becomes a fundamental issue.

Recent trends in biosensor technology reveal that four types of biosensors dominate the field of functional food safety: antibody or antigen-based biosensors, DNA-based sensors, enzyme-based biosensors, and photosynthetic protein-based biosensors.

Antibody or antigen-based biosensors can detect the presence of pesticides, veterinary drugs, steroids, pathogenic bacteria, and toxins (Ricci et al., 2007; Jiang et al., 2008; Wang and Wang, 2008; Liu and Wong, 2009). They are also known as immunosensors and can be considered as a modified version of the enzyme-linked immunosorbent assay (ELISA) test. Immunosensors detect the tiny changes that occur when an antibody binds to an antigen. Functionally, the biosensor replaces the traditional spectrophotometric detection system of the ELISA test, in order to increase the working range, speed, and sensitivity. In a simple immunosensor, the transducer surface is coated or immobilized with an antigen or antibody. An excess of a specific antibody-enzyme conjugate or antigen-enzyme is bound on the surface where the analyte solution interacts binding only to the target molecules. Unbound material is washed off and discarded. The amount of antibody-enzyme conjugate released or antigen-enzyme conjugate bound is determined directly from the transducer signal (Fig. 6).

Though immunosensors can be considered as a variant of the ELISA test, some important differences exist between the two technologies. First, the testing plates in the ELISA are generally coated with molecules of antibody; in the immunosensors, antigen coated surfaces are preferred because in the antibody-surface bond a disadvantageous orientation can occur, causing a loss of activity in the antibody or the incorrect binding of the antigen. Second, immunosensors show several advantages with respect to ELISA tests because they are faster, more sensitive, and also allow the exploration of a

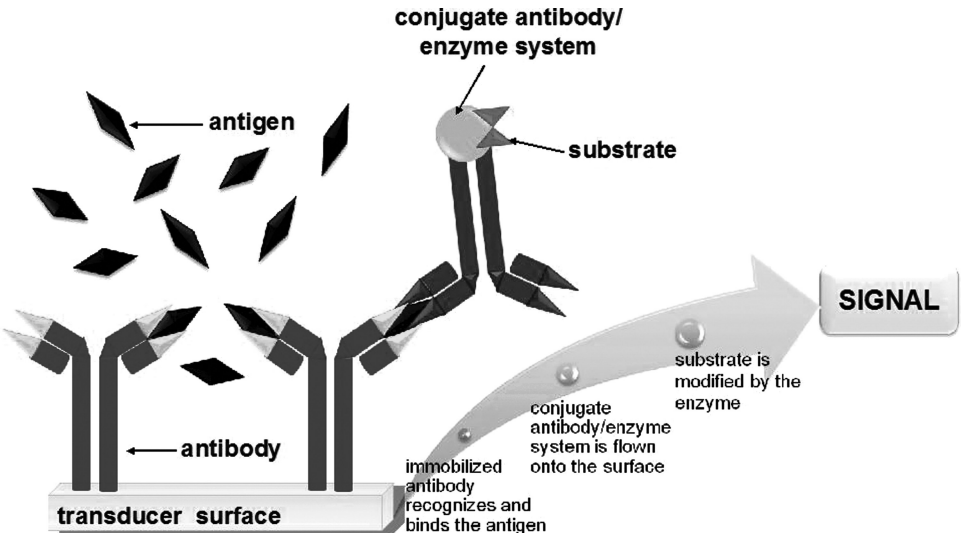

Figure 6 An example of antibody-based immunosensor: the antibody recognizes and binds the target antigen in solution producing a stable complex. After washing out the interferents, a specific antibodyenzyme conjugate complex is flown onto the system. The enzyme modifies the substrate producing a signal proportional to the amount of antigen-enzyme conjugate. Thus antigen concentration is indirectly determined. (Color figure available online.)

wider range of concentrations (Ditcham et al., 2001; Rao et al., 2006).

Apart from their involvement in phytochemical antioxidant activity recognition, DNA-based biosensors are currently used to detect pathogenic microorganisms such as *Escherichia coli* (Rodriguez and Alocilja, 2005; Sun et al., 2006; Wu et al., 2007), *Salmonella spp* (Lermo et al., 2007), *Listeria monocytogenes* (Wu et al., 2008), and *Fusarium culmorum* (Zezza et al., 2006). The DNA complementary base-pairing property underlies the recognition principle. These biosensors are, in fact, obtained by immobilizing well-defined sequences of single strands DNA on an electrode surface that in specific conditions are capable of binding DNA strands of pathogenic microorganisms. If a well-defined DNA sequence (probe) is immobilized on a surface and the complementary pathogen DNA sequence is added, the formation of a thermodynamically stable complex occurs leading to the detection and identification of the targeted organism (see Fig. 7).

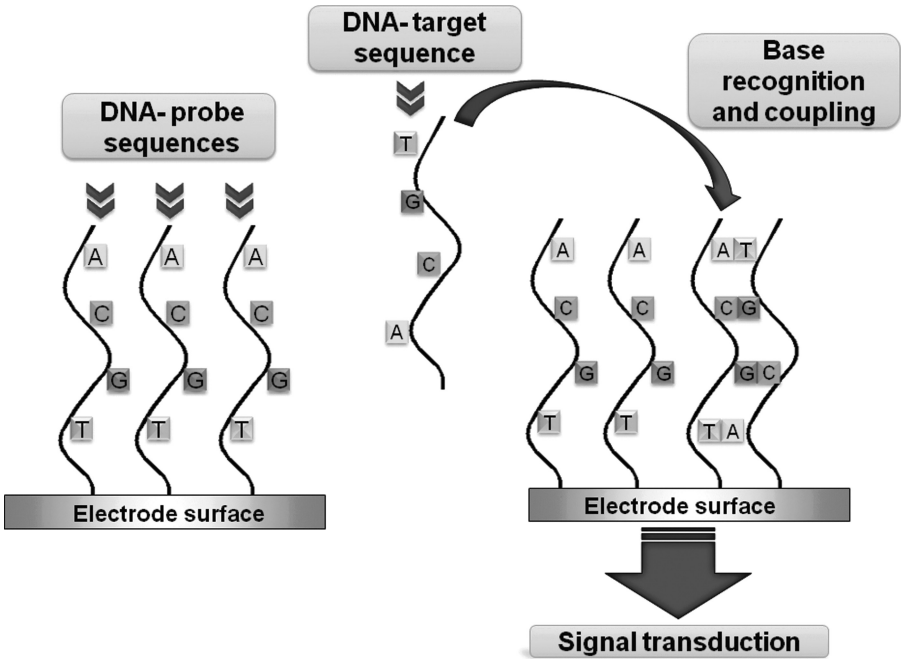

Figure 7 DNA-based biosensor to detect pathogenic bacteria. DNA probe single strands are immobilised onto an electrode surface and put in contact with the sample to analize. In the presence of complementary sequences of bacterial DNA, a linkage is established between the probe and the target DNA strand. A stable complex is formed which gives rise to a detectable signal. (Color figure available online.)

DNA-based biosensors for pathogen determination are more advantageous with respect to conventional methods such as the Polymerase Chain Reaction (PCR), the direct count of organisms, and the ELISA test; because they offer increased specificity and sensitivity (Lazcka et al., 2007) and the possibility of speeding up the detection thus avoiding excessive time-consumption involved in culturing methods.

Enzyme-based biosensors can be used both for the evaluation of the nutraceutical content and for the detection of pollutants and toxic compounds. As far as contamination is concerned, the techniques are applied mainly to the analysis of heavy metals (Berezhetskyy et al., 2008; Guascito et al., 2008), antibiotics (Fuh et al., 1988; Nishizawa et al., 1992; Meier and Tran-Minh, 1994), organophosphorus pesticides (Heo and Crooks, 2005; Luckarift et al., 2007; Pohanka et al., 2008), and carbamate pesticides (Caetano and Machado, 2008; Dan et al., 2008; Pedrosa et al., 2008).

The detection relies on two possible mechanisms; the first involves the catalytic transformation of a contaminant from a non-detectable to a detectable form while the second involves a type of detection based on an enzyme activity inhibition. In the first kind of mechanism the contaminant in the non-detectable form is the substrate of the enzyme (target analyte) and is converted into a detectable form which in turn can be reduced or oxidized on the surface of an electrode; the product is directly determined by the transducer. Examples of this mechanism include the use of tyrosinase for the detection of phenols (Morales et al., 2002; Shan et al., 2004) and the use of organophosphate hydrolase for the detection of organophosphorus pesticides (Mulchandani et al., 2001; Wang et al., 2003). (See Fig. 8a.) In the second mechanism the immobilized enzyme reacts with a known amount of substrate, and is then treated with the solution of the targeted contaminant. The toxic compound is able to inhibit enzyme activity binding to some positions around its active

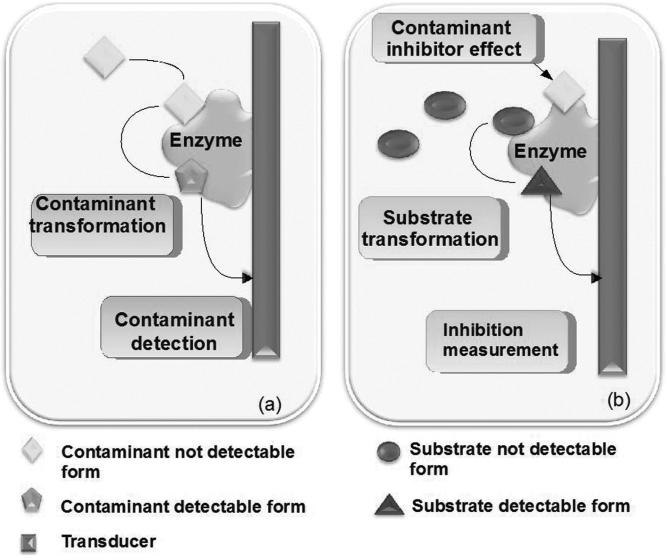

Figure 8 Enzyme-based biosensors to detect food contaminations. The detection relies on two possible mechanisms: (a) direct detection: the undetectable form of the contaminant is converted into a detectable species and is suddenly determined. (b) The contaminant acts as an inhibitor of the enzymatic reaction with the substrate. The degree of inhibition is directly correlated to the concentration of the toxyc compound. (Color figure available online.)

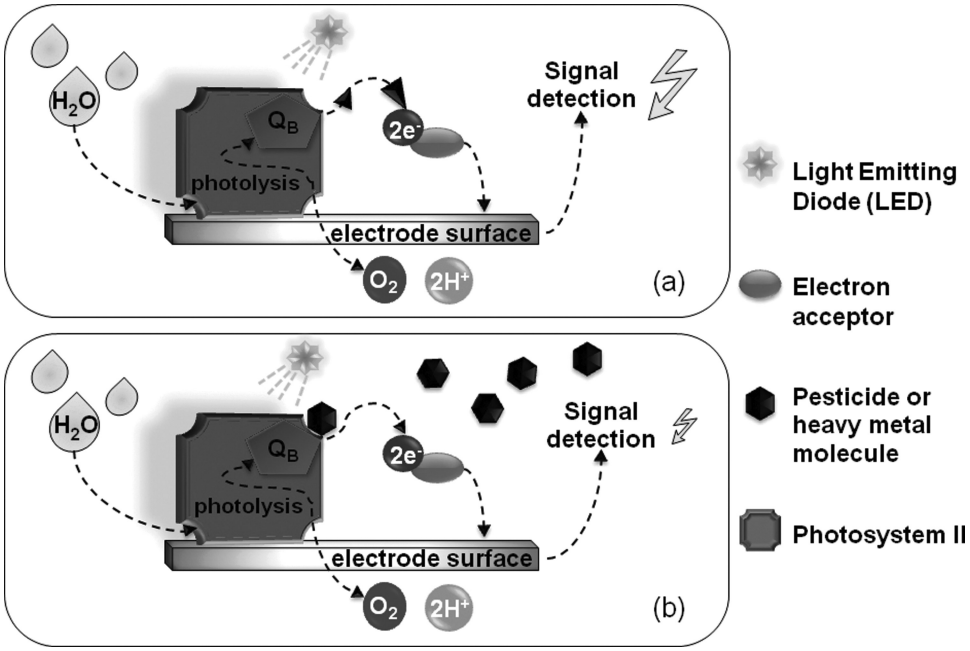

Figure 9 Photosynthesis-based biosensor for pesticides and heavy metals detection. Intact cells of photosynthetic microorganisms or plant components containing the photosynthetic protein Photosystem II are immobilized onto an electrode surface. The biomediators are stimulated by means of a Light Emitting Diode light. In absence of toxyc compounds, a transfer of electrons occurs giving rise to a detectable current (a). In the presence of pesticides the QB site of D1 subunit is occupied causing a lowering of the output signal (b). (Color figure available online.)

site. The result is a lowering of the output signal depending on the type of toxic compound and its concentration. Examples of these biosensors based on inhibitors have been reported in different applications. Cholinesterase biosensors have been reported for the detection of organophospates, carbamate pesticides, and dichlovos (Sotiropoulou et al., 2005; Campanella et al., 2007); alkaline phosphatase and glucose oxidase-based biosensors have been described for mono and divalent metal cations (Berezhetskyy et al., 2008; Guascito et al., 2008) (Fig. 8b).

Biosensors for the determination of the content of plant secondary metabolites in nutraceutical preparations have also been reported for glycoalkaloid compounds. Glycoalkaloids are a family of metabolite naturally occurring in all potato tubers and frequently found in different food matrix. Glycoalkaloids may induce gastro-intestinal and systemic effects, by causing cell membrane disruption and acetylcholinesterase inhibition, respectively. The device adopted for their detection is a pH-sensitive field effect transistor biosensor (Arkhypova et al., 2003; Dzyadevych et al., 2004).

Photosynthetic protein-based biosensors are particularly useful for pesticides and heavy metal detection and many applications are available for food. A wide variety of photosynthetic biosensors in the recent scientific literature were reported by Rouillon et al., 2006, Campàs et al., 2008, Giardi and coworkers, 2005; 2009. Photosynthesis-based biosensors use intact cells of photosynthetic microorganisms (cyanobacteria and algae) or plant components (thylakoids or isolated Photosystem II-enriched particles) as bio recognition elements. Cyanobacteria, algae, and thylakoid membranes of many higher plants contain the complex Photosystem II (PSII). PSII is a light-driven oxidoreductase protein complex enabling photosynthesis by means of a photo-induced electron transfer from water to plastoquinones. In the photosynthetic process, solar photons are captured by the antenna chlorophyll pigments surrounding the PSII reaction centers and start a cascade of oxido-reduction reactions ending with the reduction of the terminal acceptor quinone Q_B, located within the protein subunit D1 of PSII. The photosynthetic process can be monitored by immobilizing the biomaterial on an electrode surface and registering the current that flows out after photo stimulating the biomediator by means of LED. In the absence of pesticides or heavy metals, an external acceptor, added to the target solution, collects the electrons coming from Q_B quinone giving rise to an oxidation current. In the presence of pesticides and/or heavy metals, the linkage between the toxic compound and the subunit D1 of PSII causes a modification of the signal (Fig. 9) (Giardi et al., 2008, 2009).

CONCLUSION

The growing interest of consumers in the role nutrition plays on health is the primary incentive behind the success of healthy diets. People's increasing desire to be more actively involved in optimizing their personal well-being is also an additional driving force for the nutraceutical and functional food markets. Some nutraceutical compounds are of artificial origin but in recent years attention has been focused on natural compounds originating from plant secondary metabolism. Although much attention has been directed to create targeted nutrition and in vitro protocols for the mass production of secondary metabolites, little emphasis has been placed on nutraceutical activity and analysis. In this regard specific new technologies, the most appealing of which are biosensors, need to be developed and further improved.

In recent years, a wide variety of biosensors applicable to the detection of pesticides, heavy metals, pollutants, and toxic compounds in food and in the cell cultures of secondary metabolites has dominated the market, but despite the great interest in nutraceuticals only modest progress has been made. In this respect, most of the articles report the detection of phenolic compounds, a few articles on the total concentration of glucosinolates, capsaicinoids, and alliin while no specific biosensors have yet been developed for fatty acids and carotenoids. The most promising of all are the biosensors developed for the determination of antioxidant power, since ideally all plant bioactive compounds should be tested against the damaging effects of free radicals; moreover, the use of biosensors to evaluate the antioxidant properties could provide an early, non-invasive indication of some diseases linked to the presence of free radicals such as oxidative stress or its progression. In this field, various biosensors have already been manufactured using superoxide dismutase, horseradish peroxidase, cytochrome C enzymes, and DNA strands as biomediators. The stability and sensitivity of these biosensors have been demonstrated in several systems. One future development, probably with a significant impact in biosensor technology, is the study and search for new biomediators able to detect those compounds for which detection by biosensor is currently impossible. In addition, improvements in how to detect metabolites at sub-nanomolar concentrations represent an important breakthrough that will do away with the use of classical methods like HPLC-MS and GC-MS that are time-consuming and not always environmentally friendly.

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