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Mouldy fruits and vegetables as a source of mycotoxins: part 1

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Abstract

Species of *Aspergillus, Penicillium* and *Alternaria* are major contributors to fruit and vegetable decay and to mycotoxin production during various stages of pathogenesis. The mycotoxins most commonly associated with fruits and vegetables and their products are aflatoxins, patulin, ochratoxin A and *Alternaria* toxins. Naturally occurring aflatoxins are found in fruits of tropical and subtropical regions where environmental conditions support growth of aflatoxigenic aspergilli. Aflatoxins in figs and dates have been associated with Aspergillus flavus and *A. parasiticus*, ochratoxin A in figs has been related mainly to A. alliaceus, and ochratoxin A in wines and other grape-based products has been associated with *A. carbonarius* and, to a lesser extent, with A. tubingensis and A. niger. Human exposure to patulin is primarily via apple-based products, following fruit infection by *Penicillium expansum*. Attention has been drawn to patulin contamination in infant apple products, and in organic fruits versus conventional ones. *Alternaria* species, which naturally attack a wide range of harvested fruits and vegetables, are capable of producing several mycotoxins during pathogenesis. The major mycotoxins include alternariol, alternariol methyl ether, altenuene, tenuazonic acid, and altertoxin-I. Although *A. alternata* is regarded as the major producer of *Alternaria* mycotoxins, other species, such as *A. citri, A. longipes, A. tenuissima, A. arborescens*, may also produce these mycotoxins. Mycotoxin accumulation in fruits and vegetables may occur in the field, and during harvest, postharvest and storage. Factors affecting mycotoxin production include the fruit or vegetable type and cultivar, geographical location, climate, pre-harvest treatments, method of harvest, postharvest treatments and storage conditions. Considering geostatistics, knowledge of the ecology of the fungi, data on crop distribution and meteorological conditions, risk predicting maps have recently been drawn. The methodologies of detection and determination of mycotoxigenic moulds and of ochratoxin A in grape products, of patulin in apple products and of *Alternaria* mycotoxins in fruit and vegetable products, are summarised and discussed. The present review is based on the multi-author book 'Mycotoxins in Fruits and Vegetables' published by Elsevier (2008).

Keywords: *Aspergillus*, *Penicillium*, *Alternaria*, fungal detection, mycotoxin determination, risk predicting maps

1. Introduction

Fungi play a substantial role in the spoilage of fruits and vegetables, because of their pathogenicity to the harvested products. However, during the various stages of pathogenesis, some of these fungi may generate different mycotoxins, toxic to humans and animals that consume them. During recent decades a variety of fruits and vegetables that form part of our daily diet have been added to the list of products exposed to mycotoxin contamination. Since *Aspergillus, Penicillium* and *Alternaria* species are the major mycotoxigenic fungi that attack harvested fruits and vegetables, a variety of mycotoxins produced by these fungal genera during pathogenesis may contaminate the fruit and vegetable tissues. Some of these mycotoxins are highly stable during processing, therefore, although consumers will reject a visibly rotten fruit, processed fruit products may still form a significant source of these

mycotoxins, and pose a serious threat to human and animal health worldwide.

Increasing research has recently been focused on basic aspects of mycotoxins in fruits and vegetables, and a book on 'Mycotoxins in fruits and vegetables', dedicated to these aspects, published by Elsevier and edited by R. Barkai-Golan and N. Paster (2008), has just been published. This multi-author book covers the risk assessment and safety evaluation of fruit and vegetable mycotoxins, their economic aspects, factors affecting their production, and their ability to diffuse from the point of production into adjacent fruit tissues. The accumulated information on the major mycotoxins produced in fruits and vegetables during pathogenesis, including their toxicity, their sources and their occurrence, and methods for the detection and determination of the mycotoxigenic moulds and the mycotoxins produced by them, are presented. Limits set on the contents of mycotoxins in fruits and vegetables, and the corresponding regulations that have been established in many countries, and their economic aspects are discussed. Special attention has been given to chemical, physical and biological methods for suppressing the growth of mycotoxigenic moulds, methods that will indirectly lead to the suppression of toxin production, as well as to direct decontamination and detoxification of mycotoxins in fruits and vegetables and their derived products. The present review, which is based on these book chapters, is divided into two parts. The present paper is the first of these; it deals with moulds and mycotoxin formation in fruits and vegetables, diffusion of mycotoxins, risk assessment and safety evaluation, and methods for determination of mycotoxigenic fungi and their toxins. The second part, which will be published in a forthcoming issue of World Mycotoxin Journal, will deal with control of mycotoxigenic fungi and mycotoxins, effects of processing, regulations, limits and economic aspects.

2. Moulds and mycotoxin formation in fruits and vegetables

Aspergillus mycotoxins in fruits and vegetables

Aspergillus species are widespread in nature; they are regarded as soil fungi, and are also among the most common airborne fungi. They are saprophytic on a wide range of substrates, including foods and feeds, and several species are among the typical pathogens of harvested fruits and vegetables (Barkai-Golan, 2001). During their life cycles in fruits and vegetables, several *Aspergillus* species can produce various mycotoxins that are harmful to humans and animals. The major mycotoxins associated with *Aspergillus* species in fruits and vegetables are: aflatoxins, produced mainly by strains of *A. flavus* and *A. parasiticus*; and ochratoxin A (OTA), produced by *A. carbonarius* and other ochratoxigenic aspergilli. The contamination of fruits and vegetables and of their derived products with aflatoxins and OTA, both of which are carcinogenic, is of major concern for human health. In a recent review on *Aspergillus* mycotoxins, Barkai-Golan (2008a) discussed the production of aflatoxins and OTA in fruits and vegetables, with especial attention to: (1) *Aspergillus* species associated with aflatoxin production, and factors involved in its production; (2) the occurrence and levels of aflatoxins in figs, dates and other fruits; (3) *Aspergillus* species associated with OTA production, and factors involved in its production; (4) occurrence and levels of OTA in a variety of fresh and dried fruits, with particular attention to grapes and grape-derived products; (5) other *Aspergillus* mycotoxins associated with fruits.

Aflatoxins are produced mainly by strains of *A. flavus* and *A. parasiticus* in figs, dates, citrus fruits, raisins, tree nuts and olives. The optimal temperatures for fungal growth and aflatoxin production by the two high-temperature species are 35 and 33 °C, respectively (Sanchis and Magan, 2004). Neither species produces aflatoxins below 7.5 °C. Aflatoxins are often found in fruits grown under tropical and subtropical climates, where the environmental conditions support growth of mycotoxigenic aspergilli. Figs are susceptible to infection by aflatoxigenic aspergilli after the fruits ripen and soften, and such fruits serve as suitable media for aflatoxin production, probably because of their high content of carbohydrates. *Aspergillus* infection of figs has been associated with the ability of the pathogens to penetrate the internal cavity of the fruit (Doster *et al.*, 1996). The fruits are susceptible to infection during drying, but before they have dried completely. The ability of *A. flavus* and *A. parasiticus* to produce ethylene during their early growth stages was hypothesised to affect their pathogenicity and their aflatoxigenic potential. The inhibitory effect of ethylene, which is a naturally biologically active compound, was found to be exhibited at the level of transcription (Roze *et al.*, 2004). Dates grown under high humidity and at moderate temperature also suffer from aflatoxin contamination, which generally occurs at later stages of maturation. In contrast to figs, mycotoxins in dates do not appear to be naturally associated with dried fruits (Shenasi *et al.*, 2002).

Several studies have addressed OTA contamination in various fruits, such as figs, cherries, tomatoes, strawberries, apples, peaches, grapes and olives. The production of OTA in figs was related to the presence of *A. alliaceus* strains on the fruit (Bayman *et al.*, 2002). Following the finding by Zimmerli and Dick (1996) that significant levels of OTA were present in wines and grape juices, increased research has been dedicated to OTA in grapes and their products. *A. carbonarius* has been found to be the key producer of OTA in grapes and grape-derived products (Battilani *et al.*, 2004), but other *Aspergillus* species belonging to section *Nigri*, i.e. *A. tubingensis* and *A. niger*, were also found to

enhance the level of OTA in wines (Perrone *et al*., 2006). Particular attention has been drawn to OTA contents in wines because of the hazard that this mycotoxin may pose to human health worldwide. The higher levels of OTA that have frequently been recorded in red and rosé wines compared to white wines may be related to differences in the wine-making processes (Otteneder and Majerus, 2000). Dried fruits (raisins, sultanas and currants), which are regarded as health foods, can also be an important dietary source of OTA for people consuming large amounts. In general, factors affecting OTA production in grapes may include fruit maturity, the condition of the grapes, the species and isolates of the aspergilli involved, environmental conditions, and possible interactions with other fungi present on the maturing berries. Although *A. carbonarius*, the major producer of OTA, grows optimally at 30-35 °C, OTA was rarely detected at 35 °C, with little growth at <15 °C (Marin *et al.*, 2006). The occurrence and the levels of aflatoxins and OTA in their relevant fruits and fruit-derived products have been recorded (Barkai-Golan, 2008a).

Other *Aspergillus* mycotoxins associated with fruits and vegetables include: sterigmatocystin, produced by *A. flavus, A. flavipes, A. nidulans, A. ustus* and *A. versicolor*; cyclopiazonic acid, produced by *A. flavus, A. tamarii* and *A. versicolor*; aflatrem, produced by *A. flavus*; citrinin, produced by *A. flavipes, A. niveus* and *A. terreus*; and patulin, produced by *A. terreus.*

Co-occurrence of different mycotoxins in fruits, vegetables, and their products has been recorded. Examples include: the co-production of aflatoxin, cyclopiazonic acid and aflatrem by *A. flavus* isolate; the co-production of sterigmatocystin, aflatoxin and OTA in raisins infected by *A. flavus*; the co-occurrence of aflatoxin B_1 and OTA in dried figs, and the co-occurrence of cyclopiazonic acid and tenuazonic acid in tomato pulp and in tomato puree. The variety of mycotoxigenic *Aspergillus* species associated with fruit infection and the mycotoxins produced by them have been listed (Barkai-Golan, 2008a).

Penicillium mycotoxins in fruits and vegetables

Penicillium species are among the most common airborne fungi and are frequently associated with spoilage of foods and feeds. Several *Penicillium* species are highly prevalent agents of postharvest diseases, and they attack a wide range of fruits and vegetables. However, in addition to inflicting losses associated with fruit and vegetable decay, pathogenic penicillia may produce during their pathogenesis a variety of potent mycotoxins that are toxic to humans and animals who consume contaminated food. In a review of *Penicillium* mycotoxins Barkai-Golan (2008b) emphasized the following points: (1) the significance of *Penicillium* species as pathogens of harvested fruits and vegetables; (2) the variety of mycotoxins produced by *Penicillium* species, with special attention to patulin and its toxic effects; (3) factors affecting patulin contamination in fruits, with special attention to modified atmosphere storage; (4) the worldwide occurrence of patulin in fruit-derived products.

P. expansum is the major causal agent of the blue mould rot of apples, pears, plums, peaches, apricots, cherries, blackberries, melons and strawberries (Snowdon, 1990), and it is commonly found on several vegetables such as onions, garlic and cabbages (Lugauskas *et al*., 2005). However, the major source of patulin contamination is infected apples. The fungus frequently penetrates the fruit through harvesting wounds and bruises, and is responsible for a large part of the economic losses sustained during storage and shipment. Since fungal development is favoured by high humidity, the blue mould becomes a problem in fruit stored or shipped in plastic film liners. The fungus can grow at 0 °C; decay can progress, albeit slowly, at this temperature, and it spreads by contact from diseased to sound fruits during months of cold storage (Barkai-Golan, 2001). Studying the effect of CO_2-enriched atmosphere storage on *P. expansum* development and patulin production in apples packaged in polyethylene films indicated that the CO_2 which accumulated within the packaging system was very effective in suppressing fungal growth. In contrast, packaging of apples in polypropylene did not inhibit fungal growth, since the degree of polymeric branching and the pores' size allowed much less water vapour transmission through this film than through polyethylene, so that at the end of the incubation period more moisture accumulated, resulting in enhanced fungal growth (Moodley *et al*., 2002).

Patulin is a relatively simple lactone, derived mainly from species of *Penicillium, Aspergillus, Paecyclomyces* and *Byssochlamys.* The major producer is *P. expansum*, in which patulin biosynthesis was found to be mediated at the level of gene transcription (White *et al*., 2006). Other *Penicillium* species are also known to be patulin producers; among them we find several fruit pathogens such as *P. cyclopium, P. chrysogenum, P. cyaneo-fulvum, P. brevicompactum, P. crustosum, P. griseofulvum, P. olsonii, P. thomii, P. tularense* and others (Andersen *et al*., 2004; Barkai-Golan, 2008b). Exposure of humans to patulin, via consumption of infected fruits and their products, may result in severe toxicoses that include mutagenic, teratogenic, hepatoxic, nephrotoxic, neurotoxic and genotoxic effects. Although recent safety assessments have concluded that patulin is probably not carcinogenic (Speijers, 2004), the ability of patulin to cause gene mutations in mammalian cells might have a bearing on its carcinogenicity (Schumacher *et al*., 2005).

Several *Penicillium* species are capable of producing several potent mycotoxins in harvested fruits and vegetables.

Thus, *P. expansum* may produce, in addition to patulin, citrinin, penicillic acid, cyclopiazonic acid, chaetoglobosin, communesin B, roquefortine C and expansolides. The mycotoxin profile of *P. chrysogenum* includes, in addition to patulin, ochratoxin A, penicillic acid, cyclopiazonic acid and roquefortine C; *P. viridicatum* may also produce several mycotoxins such as penicillic acid, ochratoxin A, cyclopiazonic acid, citrinin, viomellein, viridicatin, xanthomegnin and other secondary metabolites; and *P. tularense* produces paspalinines, paxillines and janthitrems (Andersen *et al*., 2004, Barkai-Golan, 2008b).

Analysis of apple products confirmed that a patulin-negative sample is not always free of toxic metabolites, and it was suggested that chaetoglobosin A might be a better indicator than patulin for the presence of *P. expansum* (Andersen *et al*., 2004).

Worldwide occurrence of patulin in fruit products has been recorded, with especial attention being drawn to patulin contamination in apple products intended for infants, and to comparisons between its levels in organic and in conventional fruits, in industrial and in hand-made products, and in cloudy and in clear apple juice. The recording, in several studies, of higher patulin levels in organic than in conventional apple products (Beretta *et al.*, 2000; Piemontese *et al.*, 2005) raised the need for stricter preprocessing control of fruits grown under organic conditions.

Alternaria mycotoxins in fruits and vegetables

Various *Alternaria* species are plant pathogens that cause damage in the field, and several are postharvest pathogens of a wide range of fruits and vegetables (Barkai-Golan, 2001). *A. alternata*, which is frequently regarded as a collective group of species or as an unresolved species-group, occurs ubiquitously in the air, and is the most common species recorded in agricultural commodities. It is known as a saprophyte in food and feed products, and as a common pathogen of stored fruits and vegetables that causes postharvest economic losses (Barkai-Golan, 2001). In addition to spoiling fruits and vegetables through disease development, *Alternaria* species are also capable, during their various pathogenesis stages, of producing a range of toxic metabolites, of which a great proportion are phytotoxins that play roles in fungal pathogenicity, and several of them behave as mycotoxins, harmful to humans and animals. The optimal temperature range for *Alternaria* growth is 22-28 °C, which enables it to grow at room temperatures in various climatic regions. Its low minimal temperature of -3 °C enables it to grow under cold storage (Sommer, 1985), which is why the fungus continues to grow on cabbage, celery or other vegetables stored at 0 °C, or on certain apple cultivars stored at or below 0 °C. Since *Alternaria* mycotoxins are produced naturally in a variety of infected fruits and vegetables, and since infection may occur even under refrigeration, these mycotoxins may be considered as toxic contaminants in our everyday food (Barkai-Golan, 2008c).

A. alternata is a major pathogen of harvested tomatoes. In view of its high incidence in these fruit-vegetables, it is worth highlighting the high level of genetic diversity among isolates collected from typical black lesions of tomato (Morris *et al*., 2000). Tomato fruits stored at temperatures lower than 8-12 °C, sweet peppers stored below 7 °C, and sweet potatoes stored below 10 °C become extremely sensitive to *A. alternata* decay. Cucumbers, squashes and melons become sensitive to infection by *A. alternata* after being weakened by chilling injury or prolonged storage (Barkai-Golan, 2001). Also, *A. alternata* is one of the common pathogens of pome and stone fruits, grapes, blueberries, raspberries, persimmons, mangoes, and other fruits (Barkai-Golan, 2008c). *A. alternata* has been regarded as the most important mycotoxin-producing species. The major mycotoxins produced following infection, which belong to several different structural classes, include: alternariol ($C_{14}H_{10}O_5$), alternariol methyl ether ($C_{15}H_{12}O_5$) and altenuene ($C_{15}H_{16}O_6$), which are benzopyrone derivatives; tenuazonic acid ($C_{10}H_{15}NO_3$), which is a tetramic acid derivative; and altertoxin-I ($C_{20}H_{16}O_6$), a perylene derivative. Other *Alternaria* species are also capable of producing several mycotoxins. Thus, alternariol and alternariol monomethyl ether may also be produced by *A. tenuissima, A. brassicae, A. capsici-annui, A. longipes* and *A. solani*; tenuazonic acid may also be produced by *A. citri, A. capsici-annui, A. longipes, A. radicina* and *A. tenuissima*; and altertoxins are also produced by *A. tenuissima* and other species (Andersen and Frisvad, 2004; Bottalico and Logrieco, 1998).

Although *A. alternata* has generally been considered the most important pathogen of tomatoes, a recent study of naturally infected tomatoes indicated that the dominant *Alternaria* isolates belonged to the *A. tenuissima* species-group, followed by the *A. arborescens* species-group. The dominant *Alternaria* isolates on apples were found to belong to the *A. tenuissima* species-group, and the dominant isolates on cherries belonged to the *A. arborescens* species-group, followed by the *A. tenuissima* isolates (Andersen and Thrane, 2006). Both alternariol and alternariol monomethyl ether are mutagenic. The estrogenic potential of alternariol, its inhibitory effects on cell proliferation, and its genotoxic effect in cultured mammalian cells have been reported (Lehmann *et al*., 2006). Altertoxin I and related compounds were reported to be more potent mutagens than the alternariols and, in fact, the interest in these toxins has been focused on their mutagenic activity.

The occurrence of *Alternaria* mycotoxins, or their production following inoculation, has been recorded in tomatoes, peppers, melons, apples, blueberries, grapes, citrus fruits, olives and pecans, as well as in several processed fruits, including tomato products, apple juice, apple juice concentrate, grape and raspberry juice, prune and cranberry nectars, wine, and olive oil (Barkai-Golan, 2008c). In view of the widespread occurrence of *Alternaria* in harvested fruits and vegetables, and the high levels of toxins in some of the most important fruits for human consumption, a survey of fruit-derived products for the presence of toxins is needed, in order to determine the levels of human exposure and to evaluate the effort needed for their suppression.

Genetic diversity of *Aspergillus* and *Penicillium* species on fruits and vegetables

Emphasis was placed on the genetic diversity of *Aspergillus* and *Penicillium* species on fruits and vegetables, by Varga *et al.* (2008). Although these two genera are not considered major causes of plant diseases, species of each of them may be responsible for several rots and disorders in fruits and vegetables. The most important aspect of fruit and vegetable spoilage by these genera is, however, the formation of mycotoxins that may harm human and animal health. Toxin contamination may occur at various stages, including harvest, processing, and handling, and postharvest contamination may lead to changes in the quality and nutritional value of the fruits and vegetables. Among the fruits exposed to mycotoxins produced by aspergilli and penicillia, attention has been drawn to grapes, pome and stone fruits, figs and tree nuts.

Recent studies on grapes, wines and dried vine fruits focused on identifying the sources of OTA in several countries. The data obtained indicated that OTA contamination in Mediterranean countries and subtropical parts of Brazil, Argentina and Australia is caused by black aspergilli. These are responsible for bunch rot or berry rots and raisin mould rot, which are particularly severe in warmer grape-growing areas. Among the black aspergilli, *A. carbonarius* has been recorded as the most important source of OTA (Battilani *et al*., 2003), followed by the *A. niger* aggregate and *A. tubingensis* (Perrone *et al*., 2006). None of the uniseriate aspergilli produced detectable levels of OTA. In colder climates *P. verrucosum*, which is responsible for OTA contamination of cereals, was not found in grapes. *P. expansum*, which is a known producer of patulin and citrinin, is found on mouldy pome and stone fruits, and naturally occurring mycotoxins, such as patulin, chaetoglobosins, communesin B and roquefortine C, have been detected in windfall apples and in apples still on the tree (Andersen *et al*., 2004).

Figs are frequently contaminated by black aspergilli that colonise ripening fruits; they were identified as *A. niger, A. tubingensis, A. japonicus* and *A. carbonarius*, all of which may cause accumulation of aflatoxins and OTA (Doster *et al*., 1996). Whereas aflatoxin production in figs has been associated with *A. flavus* isolates, OTA accumulation was caused mainly by *A. alliaceus* (Bayman *et al*., 2002). Tree nuts are frequently contaminated by potentially mycotoxigenic species of *Aspergillus*, including black aspergilli, *A. flavus*, *A. parasiticus* and *A. ochraceus*. Ochratoxigenic isolates of *A. alliaceus* were hypothesized to be responsible for the occasional OTA contamination of tree nuts (Bayman *et al*., 2002).

Among the vegetables exposed to *Aspergillus* and *Penicillium* mycotoxins, attention has been drawn to tomato, onion and garlic, ginger and peanuts. *Penicillium allii* is widespread on garlic, in which it causes the typical blue mould rot. In their recent review of the molecular diversity of *Aspergillus* and *Penicillium* species on fruits and vegetables, Varga *et al.* (2008) pointed out that several species, including *P. albo-coremium*, *P. radicicola, P. tulipae* and *P. oplabrum,* are commonly found on onions. *A. niger,* the cause of the black mould in storage, as well as *A. flavus, A. alliaceus* and *A. fumigatus*, are also frequently encountered on onions, and *P. brevicompactum* was found on ginger root, on which it produced the mycotoxin, mycophenolic acid.

Aflatoxigenic *A. flavus* and *A. parasiticus* frequently occur on peanuts (Jimenez and Mateo, 2001). In fact, the contamination of peanuts by *Aspergillus* species led to the discovery of aflatoxin in the early 1960s. Other potentially toxigenic species include *A. ochraceus, Eurotium chevalieri, E. repens, A. fumigatus* and *A. terreus* (Jimenez and Mateo, 2001). Recent studies on the genetic diversity of aspergilli isolated from peanuts indicated that *A. flavus* populations exhibited higher levels of variability than *A. parasiticus* populations (Varga *et al*., 2008). Both classical and molecular breeding approaches are being used to develop peanut lines resistant to *A. flavus* infections.

Factors affecting mycotoxin production in fruits

A review by Jackson and Al-Taher (2008) was dedicated to factors that result in the accumulation of *Aspergillus, Penicillium* and *Alternaria* mycotoxins in fruits and fruit-derived products. Efforts have been made to understand the circumstances in which mycotoxigenic fungi infect fruits and produce toxins, so that conditions can be made unfavourable for toxin production. Although patulin can occur in many mouldy fruits, such as apples, pears, berries, plums, apricots and tomatoes, the major source of patulin contamination is apples with blue rot. Whole apples are not believed to contribute significantly to human exposure to patulin, since contaminated fruit is often discarded or

trimmed to remove mouldy parts before it is eaten (Jackson and Dombrink-Kurtzman, 2006). The greatest exposure to patulin comes from consumption of apple juice that was pressed from mouldy fruit.

A variety of fungi, including *Penicillium, Aspergillus, Byssoclamys* and *Paecilomyces* species, are capable of producing patulin. However, *P. expansum* is considered to be its major producer in fruits. The optimum temperature for the growth of *P. expansum* is near 25 °C, and a similar optimum (23-25 °C) has been reported for patulin production (McCallum *et al*., 2002). Although production tends to decrease with decreasing temperature, the mycotoxin can be produced at low temperatures (0-4 °C), therefore, refrigerated storage is not a practical means to inhibit patulin production. Jackson and Al-Taher (2008) listed a number of factors known to affect the production of patulin in apple products; they include apple cultivar, geographical location of the fruit, climate, preharvest treatments, methods of harvest, surface defects on the fruit, postharvest treatments, and storage conditions. Among the preharvest treatments, the trimming of mummified fruits, the effect of pre-harvest fungicidal treatments, and the presence of microorganisms naturally antagonistic to *P. expansum* have been discussed. Harvest factors such as bruising substantially increase fruit susceptibility to decay, and significantly higher patulin levels were reported in cider produced from ground-harvested apples than from tree-picked fruit. Rain during harvest facilitates increased fungal contamination, therefore fruit should be harvested in dry weather, and quickly transferred to cold storage. Strategies have been developed for reducing pathogen proliferation and patulin contamination during storage. These include removal of decayed or damaged fruits prior to packaging or processing, and maintaining sanitary conditions in fruit-packaging areas; reducing the spore load in the water system by addition of sodium hypochlorite or sodium-*o*-phenyl phenate; and the use of treatments that have shown promise in reducing patulin levels in apple juice, such as filtration, centrifugation and fermentation (Jackson and Al-Taher, 2008).

Aflatoxins are produced primarily by *A. flavus* and *A. parasiticus* in subtropical and tropical climates. A variety of fruits can support the growth of aflatoxigenic *A. flavus* isolates but aflatoxin has been found to occur naturally only in figs, dates and citrus fruits grown in regions having the high temperature conditions necessary for their growth (Karaca and Nas, 2006; Shenasi *et al*., 2002). Optimum conditions for growth of *A. flavus* and *A. parasiticus* and for aflatoxin production are 35 °C with 0.95a_w and 33 °C with 0.99a_w, respectively (Sanchis and Magan, 2004).

Dates are grown in areas where the temperature and the relative humidity (RH) are relatively high. In general, they produce aflatoxins when the fruits are stored under high RH conditions. Figs contain high levels of carbohydrates, which make them a suitable substrate for aflatoxigenic aspergilli; they become susceptible to invasion only as they ripen and soften.

Alternaria mycotoxins, including alternariol, alternariol monomethyl ether, tenuazonic acid, altenuene, and altertoxins, have been detected in a variety of fruits and vegetables, such as tomatoes, peppers, melons, citrus fruits, apples and olives (Jackson and Al-Taher, 2008). Production can occur in the field, and during harvest, transportation and storage. Since consumers reject fruits that are visibly mouldy, whole fresh fruits are not believed to contribute a significant amount of *Alternaria* toxins to human exposure. However, processed fruit products may contribute significant amounts of these toxins to the human diet. Tomatoes are particularly susceptible to invasion by *A. alternata*; the fungus requires injured or weakened tissue for penetration, and germinating spores penetrate the fruit skin through cracks or injuries, or at the calyx scar, and result in the production of *Alternaria* toxins. *A. alternata*, the most important mycotoxin-producing species, is responsible for mouldy-core rot in some apple cultivars. After colonising the flower part, the fungus grows through the open calyx tube into the core or carpel regions, during fruit development and storage. Apples with mouldy core may be used in the production of apple juice, since the mould cannot be detected by inspection of the juice. Contaminated apples may thus result in high levels of *Alternaria* toxins in processed apple products.

Considerable information on the occurrence of OTA in grapes and grape products has recently been accumulated. Preharvest, harvest and postharvest factors affect the formation of OTA in grapes and grape products, such as wines, juices and dried vine fruits. Preharvest factors include the location of the vineyard, the weather, the grape cultivar and vineyard management. Among the harvest and postharvest factors affecting OTA formation, emphasis was placed on the condition of the fruits and on the fact that fungal growth and toxin formation may be influenced by fungicides and by biological control applications. The observation that red wines tend to be more contaminated than white wines (Majerus *et al*., 2000) has led to the hypothesis that differences in the processing for the two types of wines may be responsible.

Diffusion of mycotoxins in fruits and vegetables

Mycotoxins produced in fruits and vegetables following infection may be found in their host tissues even after the fungal mycelium has been eliminated, but little has been published about the migration of mycotoxins from rotten to sound parts of the fruit or vegetable. This is particularly critical for the safety of consumers, since apparently healthy parts of rotten fruits and vegetables can be used in the

preparation of salads, juices, jams or jellies. In a review on the diffusion of mycotoxins in fruits and vegetables, Restani (2008) addressed the following subjects: (a) the role of fruit-skin integrity in mycotoxin diffusion; (b) the evidence for mycotoxin diffusion from rotten to unaffected areas; and (c) the presence of factors affecting mycotoxin diffusion in fruits and vegetables.

The role of fruit-skin integrity was discussed by Buchanan *et al.* (1975), who raised the possibility that *A. flavus* conidia might penetrate the fig skin even without the presence of visible wounds. Figs are known to lose their resistance to attack by *A. flavus* when the fruit becomes ripe and the skin softens. At this stage, however, fungal spores can penetrate the fruit skin because of the presence of microscopic organisms, such as minuscule insects or mites, which may produce invisible wounds that allow colonization and aflatoxin penetration. The presence of juice on the fruit surface at this stage has also been hypothesized to facilitate fungal colonisation. Evidence for the diffusion of the mycotoxin ochratoxin A from rotten to sound areas in fruits was given by Engelhardt *et al.* (1999), who found this toxin in cherries, tomatoes and strawberries after the removal of rotten tissue. Beretta *et al.* (2000) indicated that the concentration of the mycotoxin patulin in unpeeled portions of apples was up to 1,166 µg/kg, whereas that in the peeled part was up to 93 µg/kg. These data indicate that if apple derivatives are prepared from low-quality fruits the concentration of patulin could exceed the legal limits established by regulatory authorities (usually 50 µg/kg).

No direct correlation has generally been found between patulin concentration in apples or apple derivatives and the extent of rottenness in the fruit. In some cases very high patulin contents were detected in apples with small affected areas (Beretta *et al.*, 2000). Several studies indicated that the diffusion of patulin in apples is limited to a depth of 1-2 cm from the rotten area (Laidou *et al.*, 2001; Taniwaki *et al.*, 1992). It was concluded that contamination of apple derivatives could generally be avoided by trimming and removing a portion extending 2 cm from the rotten area. However, the risk of mycotoxin diffusion was found to depend on the consistency of the fruit or vegetable (Restani, 2008). Whereas in apples the patulin level dropped rapidly with increasing distance from the rotten area, tomatoes were easily penetrated by patulin and there was still a detectable concentration at 4 cm from the mouldy tissue (Rychlik and Schieberle, 2001). The diffusion of patulin in the flesh of pears after infection with various pathogens highlighted the importance of the specific pathogen involved (Laidou *et al.*, 2001). Patulin was detected in pear tissues at 1-2 cm from the rotten area following inoculation with *P. expansum, Stemphylium vesicarium* or *A. alternata,* but not with *A. flavus.*

3. Risks of mycotoxin contamination and evaluation of safety of fruits

Almost all the mycotoxins of main concern in fruits originate in the field during crop growth, and meteorological conditions are the key factors for the prediction of risks and the evaluation of the safety of the fruits (Battilani *et al.*, 2008). *Aspergillus* rot of grapes is particularly severe in the warmer grape-producing areas. Many efforts have been devoted to developing molecular methods to support identification of black aspergilli, and a reliable real-time polymerase chain reaction (PCR) assay was developed for detecting *A. carbonarius* in grapes (Mulè *et al.*, 2006); this is relevant in practice because *A. carbonarius* is considered to be the main species responsible for OTA presence in grapes, dried vine fruits and wine, especially red wine. Natural occurrence of OTA in grapes and wine is a problem that originates in the vineyard (Battilani *et al.*, 2004); it is formed only prior to alcoholic fermentation (Zimmerli and Dick, 1996) and is more commonly detected, and at higher concentrations, in red wines than in rosé and white wines (Otteneder and Majerus, 2000). In addition, the amount of OTA was dependent on the latitude of the producing region: the lower the latitude, the more frequent the occurrence and the greater the concentration (Otteneder and Majerus, 2000; Battilani *et al.*, 2004). Optimum temperatures for growth of *Aspergillus* section *Nigri* were found to lie between 30 and 37 °C, and optimum water activity for growth was 0.98 (Bellí *et al.*, 2004). The influence of ecological conditions on OTA production, and their possible use as OTA predictors were confirmed by field data. Battilani *et al.* (2006) succeeded in using a discriminant equation, with summation of degree-days and rain in the period between veraison and ripening as input, to classify vineyards contaminated with OTA, and this approach is also promising for prediction. Aflatoxins are synthesised by some strains of *A. flavus*, which produce essentially aflatoxin B_1 and aflatoxin B_2, and by most strains of *A. parasiticus*, which also form aflatoxin G_1 and aflatoxin G_2. Aflatoxin-producing strains of *A. flavus* and *A. parasiticus* are distributed worldwide in soil and air, and can be classified as both storage fungi and field fungi, since some strains are phytopathogenic and can infect plants in the field and then colonise harvested or stored plant products. Aflatoxins can accumulate in many important agricultural commodities, and plant products, nuts (e.g. peanuts, hazelnuts, pistachio nuts and almonds) and dried fruits from tropical and sub-tropical areas are among those presenting major risks of aflatoxin contamination. Preharvest infection and consequent aflatoxin concentration is particularly important in the semi-arid tropics, especially when drought occurs in the last 20-40 days of the season (Cole *et al.*, 1985).

Blue mould rot of apples and pears is caused by *P. expansum*, a pathogen that is generally considered to be a wound

parasite. The symptoms start with soft watery brown spots, which enlarge rapidly at 20-25 °C; blue-green spores are formed on the lesion surface in humid conditions. During storage, infection can occur even at 0 °C, but decay proceeds slowly, and usually develops rapidly only when fruits are returned to warm temperatures. The species includes strains with varied pathogenicity, as indicated by lesion diameters on fruits, and with differing capacities to produce patulin. Patulin is stable in apple juice, but when the juice is converted to cider it does not contain patulin, which is destroyed by alcoholic fermentation. The removal of contaminated apples from the initial processing line during apple juice production has to be considered seriously. The mean patulin concentration in unprocessed apples can be relevant; it can be significantly reduced by an initial water washing step, and further reduced by removal of rotten and damaged apples.

Data on world distribution of selected crops were extracted from FAOSTAT, a database managed by the Food and Agriculture Organization of the United Nations (FAO, 2008). FAOSTAT provides access to over 3 million time-series and cross-sectional data items relating to food and agriculture; it contains a full matrix of integrated and compatible statistical coverage of 200 countries over 15 years, with information on more than 200 primary products, and input items related to production, trade, resources, consumption and prices. The relevance of the roles of meteorological/ecological parameters was confirmed for all the diseases described. Thus, the risk of mycotoxin contamination in the selected fruits can be assessed, at world level, starting from the descriptions of meteorological conditions in the crop growing areas (Battilani *et al*., 2008).

During the postharvest period, environmental conditions determine the possibility of increase of mycotoxin contamination; the possibility of reducing that risk is very high in developed countries and progressively decreases in those places where human control over this part of product management diminishes. Nevertheless, in the pre- and postharvest periods, product management is optimised when knowledge on all aspects of mycotoxin-producing fungi is complete. The quality of information on the occurrence of mycotoxins and of the fungi that produce them is not the same for all products, and is certainly not equally accurate in all geographical areas of production. With the help of geostatistics, and knowledge of the ecological needs of fungi, the global level of risk was simulated, and risk maps that took meteorology, crop distribution and growing period into account, were drawn. These maps predict the risk of mycotoxin contamination in the field, irrespective of the yearly variations in meteorological data and of the effects of cropping systems. This approach cannot be very precise, because of the global dimension, the simulated approach, and the lack of cited data, but it certainly forms a good basis for evaluation of safety of fruits produced all over the world. It can be considered a prototype that could be adapted to other crops and other mycotoxins/diseases (Battilani *et al*., 2008).

Improvements to the global risk maps could be achieved through the input of more precise information regarding several aspects, such as fungal ecology, and also host crops, cropping systems, and crop cultivation areas. Furthermore, since the fungi are strictly dependent on the annual pattern of meteorological conditions, improvement of the knowledge of risks is obviously related to the availability of yearly data. Nevertheless, the maps indicate high-risk areas worldwide for all the mycotoxins considered, especially aflatoxins, and thereby indicate where close attention is required, especially when the country of origin is a third-world country where the best management practices for fruits may not be available.

4. Methods for detection and determination of fungal species and mycotoxins

Detection of *Penicillium, Aspergillus* and *Alternaria* species in fruits and vegetables

Mouldy contamination of foods, including fruits and vegetables, results in quality deterioration and, moreover, the mycotoxins that may be associated with mould development can impair human and animal health. Methods for accurate detection and quantification of the fungi and their mycotoxins are, therefore, of economic and public-health significance. Selection of appropriate methods for detecting mycotoxigenic fungi depends on factors such as the speed of the method, its accuracy, the skill level required to perform the assay, and the cost and intended use of the method. In all cases, a representative sample is important, to ensure that the results of the test can be correctly assigned to the analysed sample. In addition, valid assays for detecting mycotoxigenic fungi and mycotoxins in foods are essential for implementing control and regulatory strategies. In their recent review of the detection of *Penicillium, Aspergillus* and *Alternaria* species in fruits and vegetables, Amalaradjou and Venkitanarayanan (2008) compared the historical and conventional methods with newly developed ones.

Historically, moulds have been detected either directly, i.e. visually, or indirectly through their effects on foodstuffs. The traditional mould identification procedures are based primarily on the recognition of the characteristics of the colony and the micromorphology of the moulds, measurement of the levels of CO_2 or volatile compounds, and evaluation of the presence of fungal cell components (Cousin, 1996). Plating and dilution plating have traditionally been the main methods used to detect and to enumerate moulds in various products. The selection of growth media

is important for the success of mould detection in foods. Among the first selective media for detecting *A. flavus* and *A. parasiticus*, the two aflatoxigenic species, a differential medium that enabled the production of an orange-yellow reverse of the colonies was used (Hocking, 1997). The chemical and biochemical methods for identifying moulds are based on the detection and quantification of specific components of moulds, such as chitin, ergosterol, and fungal volatiles. A good correlation was found between the contents of chitin or its breakdown compounds, on the one hand, and mycelium growth on fruits and vegetables, on the other hand. The detection of chitin can indirectly indicate fungal activity, even after the mould has been destroyed. A wide range of techniques, including colorimetry, chromatography and infrared spectroscopy, have been used to detect chitin (Cousin, 1996).

The conventional detection techniques are, however, time-consuming, labour-intensive and require special facilities and mycological expertise. The availability of new molecular biology tools has led to the development of specific methods for rapid identification of moulds in food. Recently, several serological and molecular methods for mould detection have been developed; they are rapid, sensitive, and suitable for the detection of toxigenic moulds. These methods could be applied both in the field and after harvesting, in order to obtain real-time monitoring data on contamination, and can therefore assist in food safety assessment (Amalaradjou and Venkitanarayanan, 2008).

Methods involving immunological assays for mould detection are based on the binding of fungal antigens and their antibodies. Various sources of fungal material that are used as antigens are heat resistant and may, therefore, persist on fruit products even after the destruction of the mould. Fungal materials used as antigens include mycelium fragments, spore suspensions or their mixtures, as well as indirect fungal material, such as surface washes obtained from the growth media (Pestka *et al*., 1995). Among the immunochemical methods, the enzyme-linked immunosorbent assay (ELISA) is the most widely used. Most of the ELISA methods for detecting moulds focus on assays that use antigens from specific fungal species. Although such assays were found useful for estimating the total fungal biomass, there were difficulties in raising an antiserum that would specifically detect a single species of mould in a mixed culture (Amalaradjou and Venkitanarayanan, 2008). Various ELISA methods have been developed to detect the presence of *Penicillium* and *Aspergillus* species in fruit juices, spices and cereals, and the presence of *Alternaria* in tomato puree.

Various nucleic acid-based detection methods have been developed for the detection of major mycotoxigenic species associated with fruits and vegetables (Niessen *et al*., 2005). As the complete genome sequences of mycotoxigenic fungi become available, microarrays that use specific gene sequences are being developed. Microarray technology can be used for detecting toxigenic species belonging to the same genus or to different genera. This is important since generally a complex of species is found on fruits and vegetables.

During their exponential growth, many fungi release low-molecular-weight volatile organic compounds that result from secondary metabolism. The compositions of these volatile compounds remain qualitatively stable over a range of media and conditions, therefore an attractive and promising method for analysing mould contamination based on the use of multisensing system technology ('electronic nose') has been proposed; it enables the accurate evaluation of mould contamination in foods within minutes (Magan and Evans, 2000). A concept similar to the electronic nose but applicable to detection of moulds in liquids, such as fruit juices ('electronic tongue'), has been developed by Soderstrom *et al.* (2003). The emerging methods for the detection of mycotoxigenic moulds in fruits and vegetables have been summarised and discussed (Amalaradjou and Venkitanarayanan, 2008).

Detection and determination of mycotoxins in fruit products

Each of the steps involved in the analytical methodology of mycotoxin detection in fruit product has been described by Venâncio (2008). The methodology for mycotoxin detection in foods, including fruit products, involves a sequence of several steps: (1) sampling; (2) sample preparation; (3) extraction; (4) clean-up; final separation (5) and (6) quantification. The sampling and sample preparation are the most critical steps for achieving a precise analysis, since the material taken to the laboratory for analysis should represent the whole lot as accurately as possible. A sampling plan includes: (a) the collection of materials from individual places in the lot or sub-lot, i.e. taking incremental samples; (b) the combining and homogenisation of all the incremental samples, i.e. forming an aggregate sample; and (c) the removal of a portion of the combined material, i.e. a laboratory sample for analytical evaluation.

Determination of mycotoxins in food commodities requires a step of solubilisation of the mycotoxins with an appropriate solvent. When the food sample is a solid matrix, a solid-liquid extraction is usually carried out, but with liquid foods it may be possible to omit the liquid-liquid step, replacing it with dilution with a bicarbonate solution or a phosphate buffer solution. It is accepted that the proportion of the mycotoxin extracted, i.e. the recovery, could be determined by spiking the sample with a known amount of mycotoxin. However, spiking would lead to a non-representative sample and would provide an indication of losses at the stages after the extraction, unless the whole spiked test portion is taken into analysis. The clean-up

stage of the analytical procedure involves the separation of the mycotoxin from other co-extracted compounds. However, this stage is dependent on the type of subsequent quantification: a qualitative assay would require little or no clean-up, whereas quantitative assays would probably require more extensive clean-up. The specificity and limit of quantification will also determine the extent of clean-up required. The quantification or measurement stage takes place after the clean-up procedure when the mycotoxin is further separated from impurities.

Detection and determination of ochratoxin A in grape products

Since the first detection of OTA in wine samples by Zimmerli and Dick (1996) several surveys of divers grape products have been carried out worldwide. OTA was detected in table and wine grapes, dried vine fruits, grape juices and musts, wines and vinegars (Battilani *et al.*, 2004). In a review Venâncio (2008) emphasised that the estimation of OTA level in a bulk lot of grape products is based on concentrating incremental samples taken from the lot. Sampling has a crucial role in the analyses of whole grapes or dried vine fruits in the lots, whereas in wine and grape juices sampling is more straightforward because they are liquids. In the European Union there are regulations governing the sampling method for the official control of OTA levels in wines and other grape-derived products (EC, 2006).

Since the distribution of OTA in grapes or dried vine fruits is probably not homogeneous, an extraction process involving the use of a mechanical shaker or a blender is necessary to ensure homogeneity of the sample (Serra *et al.*, 2004). The use of acidified chloroform is a common way to determine OTA in food, since OTA binds to proteins and this solvent breaks these bonds. The use of an organic solvent is useful when the subsequent separation and quantification are by thin-layer chromatography. When further clean-up is needed, that requires the use of immunoaffinity columns (IAC), the organic solvent must be replaced with an aqueous solvent, as in the more recent methodology (Venâncio, 2008). The extraction of OTA is a difficult step, since it is not possible to calculate the relative amount of the mycotoxin extracted or solubilised by the solvent, because the amount of mycotoxin initially present is unknown. The available clean-up procedures for grape products have been summarised (Venâncio, 2008). Several methodologies have been proposed for wine, grape musts or juice, most of which are based on a common clean-up stage that uses an IAC (Venâncio, 2008). The most popular is that described by Visconti *et al.* (1999). The more specific this stage is, the fewer the clean-up and separation stages required.

Detection and determination of patulin in fruit products

Regulatory limits that have been established for patulin in apple juice to minimise human exposure have provided an incentive for the development of faster and more specific analytical methods with lower detection limits. Analytical methods for identifying and quantifying patulin in fruit and fruit juice have recently been reviewed and discussed by Sabino (2008). The extraction of patulin from fruit juice samples is usually done with ethyl acetate, and a clean-up step may use diverse techniques. The various methods for analysis of patulin in fruits and fruit juices include, among others, thin-layer chromatography (TLC), liquid chromatography (LC), gas chromatography (GC), liquid chromatography/mass spectrometry (LC/MS), gas chromatography/mass spectrometry (GC/MS) (Sforza *et al.*, 2006; Shephard and Leggott, 2000). In general, the TLC methods that predominated in the early 1970s have given way to methods based on LC, and the most common currently used method to quantify patulin in fruit products is LC with ultraviolet (UV) detection or photodiode array detection. This is the method officially adopted by AOAC International (1995) for apple juice, with a detection limit of 5 µg/l. The LC/UV procedure is routinely used for quantitative determination of patulin in apple products, but methods to confirm the presence of patulin usually include more specific detection techniques, such as mass spectrometry (MS), after LC or GC separations (Sforza *et al.*, 2006). In practice, MS has been associated with both LC and GC, a combination that enables a conclusive confirmation of the toxin.

Detection and determination of *Alternaria* mycotoxins in fruits and vegetables

Fernández Pinto (2008) reviewed the current analytical methods for the detection and determination of *Alternaria* mycotoxins in fruits and vegetables; particular attention was drawn to the extraction, purification, separation and detection stages. Alternariol (AOH) and alternariol methyl ether (AME) are usually extracted from solid foods with organic solvents, such as methanol, acetonitrile, ethyl acetate, chloroform, acidified chloroform-ethanol and methanol-hexane (Scott, 2004). Clean-up may involve treatments with sodium bicarbonate, ammonium sulphate or lead acetate solutions, and silica gel chromatography. Solid-phase extraction (SPE) columns or cartridges are used for both extraction and clean-up of AOH and AME in liquid foods, such as apple juice (Scott, 2004), and C_{18} and aminopropyl SPE columns in series are used for clean-up of apple juices and other fruit beverages (Lau *et al.*, 2003).

LC has recently replaced GC and TLC for the determination of *Alternaria* mycotoxins in food extracts. After separation by reversed-phase LC, both toxins can be detected by UV fluorescence, electrochemical detection, or MS (Lau

et al., 2003; Scott, 2004). GC/MS is a reliable technique for confirming these mycotoxins, although LC with diode-array detection (DAD) allows their confirmation without derivatisation and purity assessment of the chromatographic peaks (Scott, 2004). In a study of LC-UV analysis of AOH, AME and altenuene (ALT) in apple tissues inoculated with toxigenic *Alternaria*, the toxins were confirmed by LC-MS with an electrospray ion source (ESI) and a time-of-flight (TOF) mass spectrometer (Andersen *et al*., 2006). A method for determination of AOH, AME, and altertoxin I (ATX-I) in carrots and solid carrot-based products was developed by Solfrizzo *et al*. (2004): the commodities were extracted with a mixture of acetonitrile-methanol water at pH 3. The extracts were purified on a C_{18} column. Toxins were quantified by reversed-phase high performance liquid chromatography (HPLC) with an UV-DAD detector set at 256 nm. LC-MS and LC-MS/MS confirmatory procedures based on ESI and atmospheric chemical ionisation (APCI) with negative ion detection were applied to confirm the presence of AOH and AME in samples of apple, grape and raspberry juices, in cranberry and prune nectars, and red wine (Lau *et al*., 2003). Electrospray LC-MS/MS with negative ion detection and in multiple reaction monitoring mode offers higher sensitivity and specificity. This methodology has been applied recently for the determination of AOH and AME in red and white wines, red and white grape juices, and cranberry juices. After clean-up on aminopropyl SPE columns, the toxins were initially determined by reversed phase LC with UV detection. Positive extracts were re-analysed by LC-MS/MS negative ion in multiple reaction mode. Limits of detection were below 0.01 ng/ml (Scott *et al*., 2006). This study indicated that AOH occurs frequently at low levels in red wine.

ATX-I in mouldy tomatoes and in apples inoculated with toxigenic *Alternaria* was investigated by LC-UV DAD. In the apple tissues its presence was confirmed by LC, ESI and TOF MS. Extraction was with ethyl acetate containing 1% formic acid (Andersen and Frisvad, 2004; Andersen *et al*., 2006). Tenuazonic acid (TeA) has been found in several *Alternaria*-infected fruits and vegetables, where it accompanied AOH and AME. Extraction of TeA from food is preferably done by acidic aqueous organic solvent extraction (Scott, 2004). Tenuazonic acid was recorded together with AOH, AME, ALT, ATX-I and the phytotoxin tentoxin by LC-UV in apple tissues inoculated with toxigenic *Alternaria* strains and in naturally mouldy tomatoes (Andersen and Frisvad, 2004; Andersen *et al*., 2006). The method described by Solfrizzo *et al*. (2004) for AOH and AME in carrots and solid carrot-based products was used for the analysis of TeA, with changes in the clean-up procedure.

It seems clear that many of the methods outlined in this review can be used or modified to meet specific analytical requirements (Fernández Pinto, 2008).

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