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Ochratoxin A in liquorice products – a review

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Liquorice is a herbal medicine produced mainly in China and Iran. This plant is suspected to contain ochratoxin A (OTA), a secondary metabolite produced by fungi. Although liquorice is not included in the daily dietary of humans, the high levels of OTA reported in this product have concerned consumers. Registration of a standard method for measuring the amount of this mycotoxin in liquorice-derived products is an important challenge and requires the introduction of a reliable, simple, fast-performance and reproducible technique. This review examines studies carried out concerning the occurrence of OTA in liquorice products. Recent information regarding contaminated liquorice, the regulatory framework and methods to degrade OTA in liquorice are discussed.

Keywords: liquorice; ochratoxin A; determination; contamination

Introduction

Demands from the global pharmaceutical market for medicinal plants have vastly increased in recent years (Li et al. 2015). The root of the leguminous Glycyrrhiza crop (Glycyrrhiza glabra L.), liquorice (American English: licorice, derived from the Greek and meaning ‘sweet root’) is a plant that mainly grows in dry and grassland areas (Zhang et al. 2015). The use of liquorice in the pharmaceutical industry is significant. Traditionally, liquorice is used to treat coughs and to raise blood pressure (Schambelan 1994; Fuhrman et al. 2002). A special sweetener found in liquorice, glycyrrhizin, is up to 50 times sweeter than sucrose and can be replaced in food products (Paolini et al. 1998; Isbrucker & Burdock 2006). Liquorice is widely used as a sweetening and flavouring agent in foods, such as beverages, herbal tea, chewing gums, candies and sweets. The latter contains around 5% liquorice extract (Ariño et al. 2007a). Liquorice may also be used in cigarettes as a flavouring and casing agent (Carmines et al. 2005).

The two general forms of liquorice offered in markets are roots and extracts. The long, brown roots are usually harvested by hand after 3–4 years remaining on the field (Cheel et al. 2013). Then the residual stems and fibrous roots are removed and the plant is often sun dried (Ariño et al. 2007b). Preparing liquorice extracts is performed by chopping and then steam extraction of the roots to eliminate most of the water (Ariño et al. 2007a). The liquor is then filtered and concentrated. The final product may be a solid block or spray-dried powder.

Environmental conditions affect microbial growth on liquorice roots (Khalesi et al. 2013). Old-style harvesting, processing and storage can result in exposure to fungal contamination. The latter has three main effects: deterioration of the chemical composition, reduction of the medicinal effectiveness and risk of mycotoxin production. For reasons unknown, the presence of aflatoxin B1 (AFB1) has been reported to be a much lesser extent than ochratoxin A (OTA) on liquorice roots (Pietri et al. 2010; Wang et al. 2013). A recent report claims that only 16% of liquorice products contained AFB1, whilst most of them contained OTA, sometimes up to 990.1 ng g⁻¹ (Pietri et al. 2010). Therefore, much of the studies concerning the contamination of liquorice products have focused on OTA.

OTA is the second important mycotoxin (after AFB1) found in many plants such as wheat, corn, oat and liquorice root (Duarte et al. 2010; Vettorazzi et al. 2014), and it has been recognised as a possible human carcinogen (Group 2B) (IARC 2009; Ahmed et al. 2015). This secondary metabolite of fungi includes an iso-cumarine linked to a phenylalanine amino acid (Figure 1) (Vecchio et al. 2012; Majdinasab et al. 2015). To control OTA in liquorice, the processing steps from harvesting to market should be carefully monitored. Indeed, developing a standard method to measure the OTA in liquorice products is essential.

Up to now little studies have been carried out regarding the OTA content in liquorice derivative products. Even those, have been studied on different aspects of the issue. In this review, a connection between the existing reports concerning the levels of OTA in liquorice and its derivative products, the regulatory framework, the importance of post-harvest processing, and the operative techniques to determine OTA in liquorice-derived products are discussed in detail.

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Reports on OTA contamination in liquorice products

The first reports of the presence of OTA in liquorice (Bresch et al. 2000; Majerus et al. 2000) indicated a high percentage of contaminated samples. Bresch et al. (2000) reported that about 50% of the liquorice roots were contaminated by OTA in the concentration range of 0.3–216.0 ng g\(^{-1}\). Interestingly, homogeneous brownish samples have been reported to contain a higher amount of OTA than those bright yellow. In another study in Germany at the same time (Majerus et al. 2000), the analysis of 83 liquorice products as foodstuff or supplements in medicine indicated low levels of OTA in children’s tea (130–440 ng g\(^{-1}\)), but quite high levels in supplements (300–64 300 ng g\(^{-1}\)). More than 90% of liquorice tablets were reported to contain OTA in the range of 700–2600 ng g\(^{-1}\). Further research in Germany confirmed high amounts of OTA in liquorice products, sometimes with values above 200 ng g\(^{-1}\) (Kabelitz & Sievers 2004).

Liquorice products made in Italy have also been reported to contain OTA. While liquorice confectionery contained 0.96 ng OTA g\(^{-1}\), dried liquorice extracts had 89.6 ng OTA g\(^{-1}\) with a maximum value of 990.1 ng g\(^{-1}\), which represents an extremely high level of contaminant (Pietri et al. 2010). Given these data, the OTA intake from dried liquorice extract seems to be a risk for consumers, especially high consumers, patients and infants.

In Spain, Ariño et al. (2007a), found high levels of OTA in 30 liquorice products including food- and medicine-based samples. All the samples were found to contain OTA with concentrations up to 252.8 ng g\(^{-1}\). Dry liquorice roots contained the highest levels with a mean of 63.6 ng g\(^{-1}\), and sweets contained the lowest level with an average value of 3.8 ng g\(^{-1}\). Liquorice extract contained 16.0 ng OTA g\(^{-1}\), and solid liquorice block had 39.5 ng OTA g\(^{-1}\). Ariño et al. also notified that OTA in dry liquorice root may be shifted to corresponding tea from 1% for infusion tea up to 5% in the case of decoction tea. In another study in Spain, 75% of the liquorice hard candies purchased from public markets were reported to be contaminated with OTA at a mean value of 2.96 ng g\(^{-1}\), while for the soft candies only 39% contamination with mean value of 0.34 ng OTA g\(^{-1}\) sample was observed (Herrera et al. 2009).

The results of a study in China regarding the OTA levels in liquorice roots revealed that the highest level of contamination occurred in the samples from Jiangxi (84.4 ng g\(^{-1}\)) and the lowest from Beijing (1.4 ng g\(^{-1}\)) (Yang et al. 2010). The difference might be due to the variety of the roots and, more importantly, eco-physiological and storage conditions. This highlights the fact that the region of collection plays a significant role for the amount of OTA, showing that the sustainability of the roots against contamination may be different from place to place (Khalesi & Khatib 2011). In next section, this effect will be explained in more detail. Table 1 shows some reports about the OTA level in liquorice products.

Effect of eco-physiological factors on OTA development in liquorice products

The risk of fungal contamination of liquorice roots is increased in warm humid areas (Khalesi et al. 2013). Large quantities of liquorice roots are typically stocked for a long period before processing or transportation. A successful approach to avoid contamination of such plants is managing the storage conditions (Cairns-Fuller et al. 2005; Kapetanakou et al. 2009). The relation between OTA biosynthesis and eco-physiological factors including temperature, water activity (a_\(w\)) and type of mycobiota in the region has been studied (Khalesi & Khatib 2011). The recent report demonstrated that 22°C is the critical point at which to control the formation of OTA in liquorice roots (Khalesi et al. 2013). Below this temperature, the amount of OTA is much lower than the cited temperatures. During storage, covering the bulk of the roots should be avoided because it may increase the inlet temperature and, consequently, increase the risk of fungal growth as well as OTA biosynthesis.

Among different parameters influencing OTA formation, the mycobiota in a region of liquorice root should be particularly considered. The map of fungi distribution in liquorice production’s regions is still unknown. From different liquorice root samples collected from four locations in China, 16 fungal species such as *Penicillium, Aspergillus* and *Eurotium* were isolated (Chen et al. 2011). Among those, *P. polonicum* was the main specie in Jiangxi province (it contained the highest level of OTA), while in other regions *Aspergillus* spp., such as *A. parasiticus, A. flavus, A. versicolor* and *A. sydowii*, and *Eurotium* spp., such as *E. chevalieri, E. repens* and *E. amstelodami*, have been dominant. The same study was performed by Chen et al. (2013) who declared that *Penicillium* is the major fungi on liquorice in different regions in China with the potential for OTA production. The researchers could isolate two new *Penicillium* species, i.e. *P. glycyrrhizacola* sp. nov. and *P. xingiangense* sp. nov., in liquorice roots for the first time. *P. chrysogenum* was also reported as one of the main OTA producers on liquorice in China. Chen et al. reported that *P. chrysogenum, P. glycyrrhizacola, P. polonicum* and *A. westerdijkiae* have contaminated dry liquorice up to...
39.03 ng g⁻¹. The mycobiota in dry liquorice has not been the same as the fresh one.

Iran has no public reports concerning the mycobiota in liquorice regions. However, after observation of the OTA biodegradation on liquorice roots from the natural contamination by *A. fumigatus, A. japonicus* and *A. niger*, Khalesi et al. (2011) hypothesised that those may be the predominant OTA producers in Iranian liquorice roots.

### OTA-contaminated liquorice and the threat to health

Long-term consumption of contaminated liquorice by fungi may cause a health hazard as, for instance, chronic hypokalaemic nephropathy secondary which needs immediate attention (Vivekanand 2010). Yet, the fundamental mechanism of OTA toxicity has not been fully understood. Nevertheless, to diminish the risk, the possible intake of OTA by the consumption of contaminated liquorice was investigated. To complete the current consumption databases of EFSA concerning OTA in liquorice products, some research has been conducted on different food-based liquorice considering a normal dietary (Commission Regulation (EU) No. 105/2010).

Assuming a total mean value of 1.53 ng OTA g⁻¹ in sweets reported by Pietri et al. (2010) corresponding to a consumption of about 1.4 g liquorice sweets per day, this means a weekly intake of 12 ng OTA. For an infant with 30 kg weight, a weekly uptake of 0.4 ng kg⁻¹ body weight has been reported, which is extremely high. Another study showed that the children may consume up to 8.94% tolerable weekly intake (TWI) by the consumption of liquorice confectionary (Herrera et al. 2009). Although liquorice and its derivatives are not the dominant products in dietary intake, for high consumers of liquorice, especially children, the current amount of OTA in liquorice products is unsafe.

### Market and regulatory features

After considering the regulatory framework, liquorice producers can enter the world market for liquorice root and extraction. Liquorice cultivation naturally occurs in the wild sites between Turkey and China as well as in southern European countries (Rauchensteiner et al. 2005). China is the main producer of plant medicines with yearly trading of US$60 billion (Dubey et al. 2008). While the first herbal medicine in China is ginseng, the second is liquorice distributed mostly in the north-west of the country (Miller 1998). *G. uralensis, G. inflate* and *G. glabra* are the main varieties in China (Chen et al. 2013). It is predicted that China will become a net importer. Iran is the second largest liquorice producer in the world. A region located in the south-west of Iran, Shiraz, is the main manufacturer of Iranian liquorice with a variety of *G. violacca* (Ghahraman 1999). Due to economic sanctions imposed by the United States and European Union, Iran has faced difficulties in supplying its liquorice product to new customers. The Netherlands, Spain and Italy have been considered the main producers in Europe, with total production up to only a few 100 tonnes of liquorice roots (CBI Product Factsheet 2014). Extraction manufacturers are, however, located only in China and Iran. The average price for liquorice extract is estimated to be €6/kg, while Chinese liquorice is the most expensive due to its high content of glycyrrhizic acid (which never should be below 4%) (Cirillo et al. 2011). On the other hand, the main importers of liquorice products in the world have been reported to be Japan, Korea, Germany, the Netherlands and the United States (CBI Product Factsheet 2014).

Nowadays, the main issue for trading the liquorice products, especially those coming from Asia, is keeping the products safe from OTA. To fulfil this criterion, efforts have been made to regulate OTA levels in the liquorice (Commission Regulation (EU) No. 105/2010). For this,
OTA analysis in liquorice

Analysis of OTA in contaminated liquorice is a challenge due to the complexity of the liquorice matrix or its extract. Another problem is the distance between the exporter countries (which mainly are located in the Middle East and East Asia) and the reference laboratories (most of which are in Europe). This results in taking at least few weeks to get the certificate for the products. As the growth of fungi and OTA formation continues during the delay in analysis, many manufacturers transport their products in dry form. In addition, the cost of analysis in credible laboratories is very expensive, sometimes up to €160/sample. Given these concerns, producers still look for easier, faster, cheaper and more accessible methods to determine onsite the amount of OTA in their products. The method also must be internationally validated by a credible institute concerning reproducibility, LOD/LOQ and repeatability. To overcome this, a number of procedures have been tried in recent years to determine OTA in liquorice and derived products (Ariño et al. 2007a; Schambelan 1994; Paolini et al. 1998; Majerus et al. 2000; Isbrucker & Burdock 2006; Pietri et al. 2010; Wu et al. 2011; Wang et al. 2013; Liu et al. 2013; Khalesi et al. 2013). Many use boiled water, sodium bicarbonate or chloroform for the initial extraction of the toxin. The second step might use SPE or immunoaffinity column (IAC) as a clean-up step before final separation and quantitative analysis by LC-FLD or LC-MS/MS (Figure 2) (Trucksess & Scott 2008).

In a study by Goryacheva et al. (2007), OTA in liquorice root was extracted by boiling in water for 10 min. To improve the extraction, sodium bicarbonate was added to the matrix. The final liquid was cleaned-up by IAC before purification and quantification using LC-MS/MS. The LOQ of the method was reported to be 0.3 ng g$^{-1}$ (Bresch et al. 2000). In another study, methanol was also applied to improve the extraction procedure (Majerus et al. 2000). Given the same LOQ as previous report, liquorice sweets were successfully analysed.

To introduce a valid global method, CAOBISCO (the Association of the Chocolate, Biscuit and Confectionery Industries of the European Union) performed a protocol by sending the same samples (two liquorice powders and two liquorice pastes) to 15 European credible laboratories to assess a proposed method of OTA determination in liquorice samples (Matissek et al. 2010). The method was based on a clean-up tandem immunoassay column and consisted of three steps: extraction, clean-up and chromatography. The extraction step with and without the use of halogenated solvents was performed by the laboratories. The total analysis took less than 10 min, including 5 min for colour development. In summary, a huge diversity was observed in reported results by up to 73% deviation for the powders and 47% for the pastes. The method, however, in the case of samples with an OTA value below 10 ng g$^{-1}$ was convenient with up to 40% and 7% deviations for powders and pastes, respectively. For the levels above 100 ng g$^{-1}$, the method was not successful. The extraction method did not show any influence on OTA levels. Thus it became possible to avoid using halogenated solvents when performing the procedure. The same efforts were performed by Raters et al. (2010). They sent three different samples including one liquorice powder and two pastes to 21 laboratories (20 in Europe and one in the United States). The used novel operative HPLC method showed a very low relative standard deviation for the samples. This fast method, however, was only reliable for the contamination range of 5–100 ng OTA g$^{-1}$ sample. Since halogenated solvents were not used for the extraction step, this method has been introduced as the standard acceptable technique for CEN (European Committee for Standardization) EN 14132:2003 (Commission Regulation (EU) No. 105/2010). The LOQ for the OTA in liquorice has been reported to be 500 ng g$^{-1}$ and the mean recovery as 91% (Ariño et al. 2007a). However, the current method is quiet expensive, laborious and time-consuming.

Despite the standard methods reported above, other fast accurate methods have been also suggested. Use of a corona discharge ion mobility spectrometer (CD-IMS) in positive inverse mode was reported to be successfully...
applied for measuring OTA in fresh and stocked liquorice roots (Khalesi et al. 2011, 2013). A fast screening step was used after IAC cleaning up. An LOD equal to 0.010 ng has been reported after only a few seconds of injection. In another study, a well-developed stable isotope dilution assay (SIDA) followed by IAC was used to detect OTA in liquorice products. The results were reported to be well-matched with the ELISA technique (Lindenmeier et al. 2011).

An efficient technique to extract OTA from the matrix uses SPE columns. The SPE columns are much cheaper than the disposable IACs. They have been coupled with HPLC-MS/MS and successfully measured OTA in liquorice roots. The LOD and LOQ were reported to be 0.024 and 0.095 ng g\(^{-1}\), respectively, which highlighted this method as compatible with IAC extraction coupled with LC-MS/MS (Wang et al. 2013).

Table 1 represents different screening methods used for OTA determination in liquorice roots.

Removal of OTA by processing the liquorice
A general trend for processing the liquorice root is as follows: grinding of dry roots, extraction, filtering/centrifugation of the extract, and final concentration to achieve block liquorice (Figure 3) (Mukhopadhyay & Panja 2008). There is not so much available data for the diminishing amounts of OTA as a matter of liquorice processing. However, these effects have been investigated by determination of OTA after sorting, washing and peeling of fresh liquorice roots; as well as by a comparison of the amounts in liquorice extract and the corresponding block liquorice derived from the same raw material that had been dried (Ariño et al. 2007b). Interestingly, among different processes on fresh roots, peeling was the only one that could decline the amount of OTA below 50%. The diminishing effect of peeling was reconfirmed by another study that declared that the peeled roots contained only 2% of the OTA in unpeeled roots (Majerus et al. 2000). Although peeling of liquorice roots is considered a costly process, at present this is the only practical way to remove OTA before liquorice extraction. With the extraction process around 80% and by creating block liquorice more than 90% of the OTA has reported to be removed (Ariño et al. 2007b). Nevertheless, the concentration of OTA in these products still remains at high levels. A combination of peeling, extraction and drying has been proposed to remove OTA from liquorice.

The OTA conformation in liquorice extract was reported to tolerate temperature up to 150°C for 60 min (Ariño et al. 2007b). Therefore, it is not possible to decontaminate the liquorice products by use of heat treatment alone. In contrast, use of physical processes such as irradiation, which does not have any side-effects on health, may reduce the amount of OTA below the legal limits (Chen et al. 2011). Nevertheless, keeping the roots in clean storage and processing them by taking into account the hygienic conditions may prevent the OTA becoming widespread.

There are no reports concerning the decontamination of OTA in liquorice-derived products after processing steps. The general methods for decontamination of OTA in food products have been reviewed by many authors, such as Amezqueta et al. (2009) and Quintela et al. (2013).

Conclusions
Liquorice is a plant with plenty of applications in the food and pharmaceutical industries. This crop may be contaminated by fungal OTA, a carcinogenic mycotoxin, during growing and storing. In the European Union the maximum amounts of OTA in liquorice root and extract have been authorised at 20 and 80 ng g\(^{-1}\), respectively. Nevertheless, a valid method to quantify this toxin in liquorice is still a challenge. Use of IAC and SPE clean-up methods followed by LC has been proposed for the determination of OTA in liquorice products. However, those methods are time-consuming, laborious, costly and have restricted operation in only a few laboratories, which mostly are located in Europe. Besides, decontamination of OTA in liquorice is also a very challenging topic. Peeling,
extraction and dehydration have been reported to reduce the amount of OTA, though the thermal stability of OTA is an obstacle for heat deterioration of this mycotoxin in liquorice roots and derived products. It seems that many more studies on the determination and decontamination of OTA are required to lessen the concerns about OTA in liquorice marketing.

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