1   **Retrospective and Prospective Look at Aflatoxin Research and Development from a**

2   **Practical Standpoint**

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13
14   **Abstract**

15   Among the array of structurally and toxicologically diverse mycotoxins, aflatoxins have attracted

16   the most interest of scientific research due to their high toxicity and incidence in foods and feeds.

17   Despite the undeniable progress made in various aspects related to aflatoxins, the ultimate goal

18   consisting of reducing the associated public health risks worldwide is far from being reached due

19   to multiplicity of social, political, economic, geographic, climatic, and development factors.

20   However, a reasonable degree of health protection is attained in industrialized countries owing to

21   their scientific, administrative, and financial capacities allowing them to use high-tech agricultural

22   management systems. Less fortunate situation exists in equatorial and sub-equatorial developing

23   countries mainly practicing traditional agriculture managed by smallholders for subsistence, and

24   where the climate is suitable for mould growth and aflatoxin production. Accordingly, it is difficult

25   to harmonize the regulatory standards of aflatoxins worldwide, which prevents agri-foods of

26   developing countries from access to the market of industrialized countries. To tackle the multi-

27   facetted aflatoxin problems, actions should be taken collectively by the international community

28   involving scientific research, technological and social development, environment protection, etc.

29   International cooperation should foster technology transfer and exchange of pertinent technical

30   information. This review presents the main historical discoveries leading to our present knowledge

31   on aflatoxins and the challenges that should be addressed presently and in the future at various

32   levels to ensure higher health protection for everybody. In short, it aims to elucidate where we

33   come from and where we should go in terms of aflatoxin research/development.

34
35   **Key words:** Aflatoxins; liver cancer; public health risk; foods and feeds; control means, climatic
36   change

1   Introduction

Moulds have long been raising health issues in humans, animals as well as in plants; and they continue to be of major concern to public health and a considerable burden to economy worldwide [1]. In addition to mycoses, they produce a myriad of poisonous toxins (mycotoxins) causing debilitating acute and chronic diseases in humans and animals. Moulds and their toxins have been the source of recurring disasters throughout the history of the mankind, but the identification of moulds as living organisms able to grow and interfere with human, animal, and plant health remained a mystery for a long time. When no causal relationship between the presence of pathogenic moulds and their adverse health effects could be established, superstitions and religious beliefs replaced rational and realistic explanations.

The awareness of mycotoxins impact on public health and economy has drastically increased since the early 1960s with the discovery of aflatoxins following an outbreak that caused a massive poultry death in England [2]. The incident has stimulated collaborative research between scientists, avian producers, and professional organizations from different countries to identify the causal agent and reveal its toxicity features in an attempt to design efficient control and preventive means. Impressive progress has been made within the first years following the poultry intoxication outbreak, leading not only to the precise identification and chemical characterization of the mycotoxin involved, i.e., aflatoxins, but also to the development of reliable analytical techniques and toxicity tests, which contributed significantly to the emergence of mycotoxicology as a standalone discipline.

Since then, scientific research has been intensified to identify as many as possible mycotoxins with their producing fungi and the ecological conditions for their production. Studies on the toxicity patterns of mycotoxins and the extent of food and feed contamination have also

60   been widely conducted to determine the exposure and associated health risks. While the

61   carcinogenicity of aflatoxins to animals was established concomitantly with their discovery, a

62   heated debate has been ongoing for more than two decades before the International Agency for

63   Research on Cancer (IARC) recognises aflatoxin B1 (AFB1) as a human carcinogen [3].

64   The advent of powerful analytical tools and computer-assisted equipment at the fine point

65   of sophistication [4] accelerated the detection and characterization of new mycotoxins leading to

66   a fulgurant increase in the number of the naturally produced mycotoxins and toxic metabolites

67   derived thereof. Newer types/forms of mycotoxins continue to be identified as natural fungal

68   secondary metabolites (emerging mycotoxins) or as modified mycotoxins (conjugated and masked

69   mycotoxins), representing further challenges for their detection, toxicological properties, and the

70   evaluation of associated health risks on humans and animals. These mycotoxins may interact with

71   aflatoxins when present in the same food or feed to increase or decrease the overall toxicity, which

72   may lead to re-think control means and/or regulatory standards. Despite the scientific progress in

73   the field of mycotoxicology, in general, there is still much to do to reach the ultimate goal of

74   developing efficient and cost-effective preventive and control means ultimately aiming at

75   reduction of the associated health risks to the lowest possible level.

76   This review retraces step-by-step the historical events and discoveries that have led to the

77   present knowledge on aflatoxins, as the most potent and widespread mycotoxins attracting the

78   highest attention of food safety specialists, food and feed producers, stakeholders, and consumers

79   as well as international and regional organizations. It also discusses the many challenges that

80   remain to be overcome at the scientific and practical levels in order to reduce their present and

81   future economic and health burden.

82   ## 2   Before and after aflatoxin discovery

83   *2.1   Early knowledge of fungal toxigenicity*

84   Crop contamination with toxigenic moulds and associated health problems are as old as

85   the settlement of the mankind with the advent of the first agricultural revolution in the new stone

86   era (Neolithic age) some 12,000 years ago [5]. Plant domestication and the practice of agricultural

87   activities raised the need for storage of harvested crops, especially grains, which were exposed to

88   uncontrolled fungal contamination due to the lack of technical knowledge and the use of

89   inadequate facilities. Consequently, unexplained devastating disease outbreaks struck repeatedly

90   causing massive deaths of humans and domestic animals without suspecting the implication of

91   contaminated grains [6-7]. While infested grains were described in Sumerian clay tablets as early

92   as 1900-1700 BC, the first authenticated Chinese writing reporting on the use of ergot in obstetrics

93   dates back to 1100 BC [7]. An Assyrian tablet describing fungal contamination of grains as a

94   "*noxious pustule in the ear of grain*" 600 years BC was probably the first reference to a cereal-

95   borne toxic principle, although it was considered as a cereal disease with no inference to its living

96   nature [7-8]. Because of the ignorance of the microbial world and its relation to the safety of foods

97   and feeds, these diseases were believed to be god's punishment for sins or demonic possessions.

98   The notorious "witch-hunt" trials of Salem (Massachusetts, USA) in the late 17th century, where

99   innocents were executed on an account of being the spiritual instigators of a mysterious deadly

100   disease [9], retrospectively anticipated to be ergotism [6], is an illustration of the prevailing mis-

101   interpretations of such diseases. Ergotism caused by the ingestion of alkaloids via ergot-sclerotia

102   contaminated rye grains or bread derived thereof is a significant milestone of such events whose

103   causative agent has long remained a mystery. In the Middle Ages, recurrent epidemics of ergotism

104   have been documented mainly in continental Europe with an accurate description of two distinct

105   forms of the disease: convulsive and gangrenous [10]. Although the number of victims of these

106   epidemics remains uncertain due to confusion between the gangrenous form of ergotism and

107   bubonic plague, it is generally admitted that deaths were counted by thousands [8,10]. While the

108   causal link between the disease and ergoty rye grains was established in 1630, the specific

109   responsible agent was only identified in 1853 as *Claviceps purpurea* by the French mycologist

110   Louis-René (Edmund) Tulasne [11]. About a decade later, a preliminary characterisation of *C.*

111   *purpurea* toxic principles was reported by Wenzell [12] who separated two impure fractions

112   (ergotina and ecbolina) from spurred rye and demonstrated their alkaloid nature as well as some

113   physiological effects they exert on humans. By the turn of the 20th century, a pure ergot alkaloid

114   (ergotinin) was obtained in a crystalline state, although later reported to be devoid of

115   pharmacodynamic activities [13]. By the first half of the 20th century, a series of studies pioneered

116   by Barger and Carr [14] purified and characterized different bioactive ergot alkaloid molecules

117   demonstrating that they are lysergic acid derivatives [8,15-16].

118   A number of other mycotoxicosis episodes have been recorded throughout the history in

119   different regions of the world where they induced high mortality in human and animals, thereby

120   affecting dramatically the economy and social life. Acute cardiac beriberi, alimentary toxic aleukia,

121   equine leucoencephalomalasia, stachybotrotoxicosis, and Balkan endemic nephropathy are some

122   of the remarkable outbreaks associated with fungal intoxications that have been documented. Apart

123   from the negative social and economic impact of these diseases, they have paved the way to our

124   current knowledge on mycotoxins, as the driving force for joint efforts to understand the diseases,

125   determine their aetiologies, and eventually design efficient treatments or control means. As a result,

126   some of the latter diseases have been eradicated or are well controlled to minimize their impact on

127   public health. The research history of major mycotoxicoses and related discoveries were

128   thoroughly reviewed previously [3,6,17], and they will not be further considered in this review,

129   which will focus henceforth on aflatoxins.

130   *2.2   Aflatoxins as a group of chemically related poisons: The birth of mycotoxicology*

131   The discovery of aflatoxins in the 1960s following an outbreak of "turkey-X" disease, so-

132   called because of its unknown nature and aetiology, was the turning point that has led to the

133   emergence of modern mycotoxicology discipline. In 1960, the disease appeared in poultry farms

134   located in a circumscribed area of London (UK) where they caused the death of more than 100,000

135   turkeys fed on rations with imported Brazilian groundnut [2]. To tackle the incident, W.P. Blount,

136   director and chief poultry advisor of the affected farms, conducted intensive field and laboratory

137   investigations whose findings and conclusions were published the following year [2]. Although

138   his endeavour failed to determine the causative agent involved, it provided a sound basis for peer

139   researchers to make fairly rapid progress in reaching the goal. Notably, the author established the

140   causal link between the toxicity of the feed and the disease; he accurately described the symptoms

141   and lesions induced in target organs (mainly the liver); and he ruled out the involvement of

142   infectious agents (bacteria and viruses) as well as potentially toxic chemicals commonly

143   encountered in poultry feed as contaminants, as ingredients or additives, or as a result of deceptive

144   practices. Meanwhile, the disease continued to cause deaths among turkeys, ducklings, and

145   pheasants in the same area of London, specifically targeting the farms supplied from mills run by

146   the same company that incorporated the Brazilian groundnut in feed formulations [18]. This

147   observation urged interested parties to focus on the Brazilian nut as the main suspect. Indeed, its

148   toxicity was further confirmed by feeding trials on turkeys, ducklings, chicken, cattle, sheep, pigs,

149   and rats which, upon post-mortem examination, showed similar lesions, with the liver being

150   consistently and most severely damaged [2,18-23]. However, the animals displayed different

151   sensitivities in terms of the toxic doses, mortality rates, onset of symptoms, and severity of lesions

152   depending on the species and the age, with ducklings being the most sensitive and rats the most

153   resistant; and the younger the animals, the more sensitive they were. Interestingly, among poults,

154   chickens were the most resistant, as was substantiated by feeding trials and by the observation

155   that they were the least massively affected by the outbreaks [20]. The outcome of these studies has

156   definitely established the toxicity of the Brazilian groundnut meal and its implication in the X

157   disease, as was corroborated by the improvement of the situation when the Brazilian groundnut

158   was withdrawn from feed formulations [23-24].

159   A significant achievement that paralleled the above findings, was the development of a

160   reliable and fairy rapid biological technique for quantitative toxicity testing, owing to the

161   successful preparation of a concentrated suspension of the toxin from the incriminated groundnut

162   samples [22,25]. This afforded the availability of suitable material which could be conveniently

163   administered *per oral* or intraperitoneally to animals at specific doses to gauge the extent of

164   toxicity and have a response in relatively short time. The preparation procedure consists of

165   extracting the toxic groundnut in hot methanol and suspending the dry residue derived therefrom

166   in water for fractionation with chloroform. The dried residue of the chloroform fraction was then

167   suspended in a methanol-water solution, defatted with petroleum ether, and distilled to remove

168   methanol. A 250-fold concentrate of active toxin was hence recovered as an aqueous suspension

169   after methanol evaporation, which could be orally administered to ducklings at different doses for

170   quantitative toxicity testing [22]. Nonetheless, the toxic principle in the groundnut remained

171   unknown at this point, and the only indication to its chemical nature was that it differs from the

172   naturally occurring alkaloids of ragwort plant (*Senecio*) [25]. The latter toxicant was known to be

173   associated with poultry poisoning that elicits indistinguishable symptoms and lesions from those

174   of the "X" disease [26]. Since groundnut samples from different origins were not all toxic [23,27]

175   and most of the toxic samples contained dead or live hyphae [18,24,28], it was assumed that the

176   toxin is a fungal metabolite rather than an inherent poisonous constituent of groundnut [22].

177   In the meantime, the X disease was reported in Kenya as the cause of substantial losses in

178   ducklings fed on rations containing groundnut exhibiting evident mould growth [20]. Mould

179   contaminants were isolated and sent to the Central Veterinary Laboratory at Weybridge (England)

180   where they were identified as *Aspergillus flavus* Link ex Fries [18]. Selected isolates were grown

181   in laboratory media and the cultures extracted with chloroform before separation by paper

182   chromatography developed in *n*-butanol-acetic acid. One out of 8 tested extracts showed a spot

183   with an R_f of 0.7 fluorescing in blue under UV light. The corresponding extract was orally

184   administered to a-day-old ducklings that it killed within 24 h eliciting the typical symptoms and

185   liver damage of the X disease [18]. Given the low purity of the extract, it was suggested that the

186   blue fluorescing spot contains more than one toxic metabolite [29]. This fraction was then further

187   resolved by thin-layer chromatography (TLC) on alumina plates revealing two distinct fluorescent

188   spots under UV light; one fluorescing in blue at an R_f of 6.0 and the second fluorescing in green at

189   a slightly lower R_f [30]. It was, therefore, concluded that at least two toxic metabolites of *A. flavus*

190   are involved in the X disease and were designated "aflatoxins" referring to the producing mould

191   species ("a" for *Aspergillus* and "fla" for *flavus*), and the letter B or G was added to differentiate

192   between the types of toxins on the basis of their fluorescence under UV light ("B" for blue and "G"

193   for green).

194   The improvement of separation methods by chromatography has played a pivotal role in

195   the progress of research on aflatoxins. These techniques provided key tools to prepare aflatoxins

196   in a crystalline state for more precise characterization of their chemical properties and reliable

197   toxicity testing [29,31]. Crystalline aflatoxin was obtained by cultivating toxigenic *A. flavus* under

198   controlled conditions in synthetic laboratory media or in sterile crushed groundnut [29-30]. In both

199   of the latter studies, recovery of crystalline aflatoxin from the cultures in synthetic media was

200   relatively easy and straightforward. It could be achieved by direct extraction of the culture with

201   chloroform, after which the extract is concentrated and chromatographed on silica gel column in

202   chloroform/ethanol. The fraction fluorescing in blue under UV light was dried and the residue

203   recrystallized from Chloroform/ethanol. Conversely, the recovery of crystalline aflatoxin from the

204   cultures in crushed groundnut was rather tedious, as it required different steps of extraction,

205   fractionation, and purification using various solvent systems and chromatography techniques. To

206   this end, Nesbitt, O'Kelly, Sargeant [30] followed the previously described procedure leading to

207   the 250-fold concentrate suspension [22] and continued by extracting the suspension with

208   chloroform, concentrating the chloroform extract and separating the concentrate by column

209   chromatography on silica gel. The fluorescent fraction was recovered from the column, dried *in*

210   *vacuo* and the residue recrystallized from benzene or methanol. Aflatoxin B (AFB) and aflatoxin

211   G (AFG) contained in the resulting crude crystalline aflatoxin were then separated from each other

212   by 200-transfer counter-current distribution in an appropriate solvent system. The LD_{50} of AFB

213   was determined to be 20 µg on a-day-old ducklings. Elementary, mass spectroscopy, and

214   absorption spectra analyses assigned to AFB and AFG the respective formula $C_{17}H_{12}O_6$ and

215   $C_{17}H_{12}O_7$, molecular weights 312 and 328, and maximum fluorescence peaks 429 and 450 nm. A

216   similar approach was developed simultaneously by Van Der Zijden, Koelensmid, Boldingh [29],

217   with some differences in solvent systems and purification steps, to yield amorphous colourless

218   platelets designated FB1 whose toxicity was confirmed on a-day-old ducklings giving an LD_{50} of

219   30-50 µg [29]. Physicochemical characterization of FB1, revealed striking similarities with the

220   AFB identified previously by Nesbitt, O'Kelly, Sargeant [30], notwithstanding some differences

221   in the physical properties (e.g., melting temperature and Infra-red, nuclear magnetic resonance,

222   and ultraviolet absorption spectra) that were explained by impurities possibly present in each of

223   the preparations. Indeed, a chloroform extract concentrate obtained from liquid culture of toxic *A.*

224   *flavus* and fractionated by ascending TLC resolved into at least 12 different compounds [31]. Five

225   of these compounds, three fluorescing in dark-blue and two in blue-green under UV light, tested

226   hepatotoxic to ducklings. The authors noted that additional discrete fluorescent compounds may

227   be present in the extracts, but they should be separated by as yet to be developed techniques. In an

228   attempt to improve the technique, a concentrate of chloroform extract obtained according the latter

229   procedure [31] was derivatized by treatment with Girard's T reagent and its decomposed derivative

230   fractionated by two-dimensional TLC showing a complex pattern of fluorescent spots [32]. Two

231   of these spots fluorescing in blue-violet and in violet under UV light, designated FB1 and FB2

232   respectively, prove to be highly toxic to duckling embryos (100% mortality). FB1 was

233   recrystallized from chloroform/ethanol yielding a pure crystalline toxin for further characterization.

234   Results of magnetic resonance, elementary analysis, and mass spectrometry determined the MW

235   and formula as 312 and $C_{17}H_{12}O_6$, respectively. Comparison of the results from different studies

236   on the characterization of aflatoxins revealed that FB1 [32], B [30], and B1 [31] isolated directly

237   from toxic Brazilian groundnut, in addition to FB1 purified from a culture of *A. flavus* in liquid

238   media [29] were all the same toxin, presently known as aflatoxin B1 (AFB1).

239   To further improve the purity of aflatoxins, a crude crystalline aflatoxin prepared from a

240   culture of toxigenic *A. flavus* in sterile crushed groundnut was fractionated by column

241   chromatography and purified in chloroform containing 0.25% methanol. After removal of

242   methanol, 4 closely related aflatoxins were recrystallized separately from different solvents (Table

243   1). These aflatoxins were shown to be hepatotoxic to a-day-old ducklings to different extents, with

244   AFB1 being the most toxic (LD_{50} of 30 μg) followed by AFB2 (LD_{50} of 60 g), while AFG1 and

245   AFG2 were far less toxic and a dose higher than 200 μg was necessary to kill the birds after 4 days

246   [33]. This study also established the main chemical properties of the different aflatoxins (Table 1)

247   and demonstrated that AFB1 and AFG1 are dihydro-derivative precursors of AFB2 and AFG2,

248   which could be prepared *in vitro* by chemical hydrogenation of their respective precursor

249   molecules. Based on the above chemical characterization complemented by other chemical

250   reactions and nuclear magnetic resonance (NMr) spectrum, Asao, Buchi, Abdel-Kader [34] and

251   van Dorp, van Der Zijden, Beerthuis [35] illustrated the molecular structures of AFB and AFG as

252   difuranocoumarin derivatives.

253   **Table 1**: Main physicochemical properties of aflatoxins as originally determined by
254   Hartley, Nesbitt and O'Kelly [33].

Aflatoxin	Crystallization solvents	Fluorescence under UV light (360 nm)	Fluorescence spectrum		MW	Melting point °C	Chemical formula
			Excitation wave length (nm)	Emission wave length (nm)			
AFB1	Trichloroethylene /chloroform	Blue	365	425	312	265-270	$C_{17}H_{12}O_6$
AFB2	Methanol	Blue	365	425	314	305-309	$C_{17}H_{14}O_6$
AFG1	Methanol	Green	365	450	328	247-250	$C_{17}H_{12}O_7$
AFG2	Ethanol	Green-Blue	365	450	330	237-240	$C_{17}H_{14}O_7$

255

256   To study the fate of aflatoxins after ingestion, a series of trials was conducted on different

257   animals given aflatoxin-containing rations and their excretion fluids (urine and milk), organs (e.g.,

258   liver, kidney), and product (eggs for poults) were analyzed for the presence of the aflatoxins or

259   their derived metabolites [36-40]. Allcroft and Carnaghan [36] first reported that milk drawn from

260   cows fed on toxic groundnut elicited a toxicity pattern in ducklings similar to that of the ingested

261   toxic feed, and most of the toxin that they called "milk toxin" precipitated with the casein fraction

262   of the milk. To further explore this observation, de Iongh, Vles and van Pelt [40] analyzed milk

263   powder obtained from cows fed on concentrate rations containing highly toxic groundnut. Samples

264   of the milk powder were extracted with different solvents and the final extract subjected to TLC

265   on silica gel. The chromatoplates showed the presence of a spot with the same blue-violet

266   fluorescence as AFB1 but with a much lower R_f of 0.34. The blue-violet fraction was further

267   purified and tested for toxicity on ducklings where it induced the typical bile duct proliferation of

268   aflatoxin lesions. Apart from its secretion in milk, the latter toxin was detected in the liver, kidney,

269   and urine of sheep that had been administered a mixture of AFB1, AFB2, AFG1, and AFG2 [38].

270   The "milk toxin" was then permanently assigned the designation "aflatoxin M" (AFM) referring

271   to milk where it was first detected. For further characterization, Holzapfel, Steyn and Purchase [39]

272   extracted AFM from sheep urine and subjected the extract to paper chromatography where it

273   resolved into 2 fractions; the first fraction (M1) had the same R_f and fluorescence as the parent

274   AFM, while the second (M2) fluoresced in violet and had a lower R_f of 0.23. The same study

275   revealed that the newly characterized aflatoxins M1 (AFM1) and M2 (AFM2) are hydroxylated

276   metabolites of AFB1 and AFB2, respectively.

277   Additional aflatoxin types have since been discovered either as naturally occurring fungal

278   metabolites or as derivatives generated from the metabolism of parent aflatoxins that accumulate

279   in organs and/or are secreted in body fluids. This was the case of the aflatoxin hemiketals $AFB2_a$

280   and $AFG2_a$, the respective hydroxyderivatives of AFB1 and AFG1, which were first shown not to

281   be toxic to a-day-old ducklings after administration of 1.2 mg of each toxin [41]. In contrast, a

282   subsequent study confirmed the toxicity of $AFB2_a$ to ducklings and bacteria, although to a

283   significantly lower extent compared with AFB1 [42]. Aflatoxin B1 could also be metabolized by

284   monkey and excreted in the urine in a demethylated form as aflatoxin P1 (AFP1), now being used

285   as a biomarker to assess the exposure of humans to AFB1 [43]. At present, more than 18 types of

286   aflatoxins with different chemical properties and toxicity patterns are known, among which at least

287   13 types are naturally occurring in foods and feeds that had supported the growth of aflatoxigenic

288   moulds [44-45].

289   *2.3   Causality between aflatoxins and liver cancer*

290   While scientific research has been focused on the detection and characterization of

291   aflatoxins during the early 1960s, a survey on the incidence of various types of cancer in Africa

292   indicated that the rate of hepatocellular carcinoma (HCC) was particularly high in specific regions

293   of the continent [46]. The author of the survey speculated that fungal toxins including the, by then,

294   newly discovered aflatoxins, are possible causative agents. This speculation was supported by the

295   earlier observation that peanut was incidentally commonly consumed in the regions where liver

296   cancer was frequently diagnosed [47-49]. On the other hand, peanut was known to be usually

297   contaminated with aflatoxins at levels frequently exceeding 2 mg/kg and, in some instances,

298   reaching 20 mg/kg in the high risk regions for liver cancer [50]. The suspicion of the association

299   between peanut consumption and liver cancer was also supported by health issues that arose from

300   the implementation of the United Nations Food and Agricultural Organization (FAO) initiative to

301   control kwashiorkor in African countries. In this initiative, a-year-old malnourished children were

302   fed meals supplemented with peanut as a protein source for 10 months at the daily rate of 70 g for

303   an initial period of 4 months and 140 g for the next 6 months [51-52]. Two of these children were

304   clinically evaluated 4 years after termination of the initiative and found to carry chronic liver

305   damage [51]. Preserved remnants of peanut supplement were retrospectively analyzed for

306   aflatoxins and found to contain between 0.5 and 1.0 mg of aflatoxin/kg meal, thereby exposing the

307   children to a daily intake of 35 to 140 µg aflatoxin [49]. As a consequence, the FAO established

308   a provisional limit of 30 µg/kg of peanut supplements on the basis of risk/benefit considerations

309   as a preventive measure from chronic liver intoxication [53]. The first acute toxicity of aflatoxins

310   causing liver failure was reported in 1967 in Uganda following the death of a teenager who had

311   been regularly eating mouldy cassava contaminated with 1700 µg/kg of aflatoxins [54]. The post-

312   mortem histopathological examination showed that the child's liver tissues had undergone

313   identical changes to those described in a monkey treated experimentally with aflatoxins [55].

314   By the end of 1960s, numerous epidemiological studies and animal trials have reported on

315   the relationship between the exposure to aflatoxins and different types of liver cancer. Relevant

316   reports were critically reviewed in the first volume of the IARC which recognized the widespread

317   aflatoxin-contamination of marketed and cooked foods in developing countries, mainly in the sub-

318   Saharan Africa and Southeastern Asia regions. The IARC then considered that the pertaining

319   studies provide a "circumstantial evidence" for the association of aflatoxin dietary intake with liver

320   cancer in humans [56]. Nonetheless, the direct causal relationship was explicitly excluded,

321   essentially because of two confounding factors: (i) the concomitant presence of other mycotoxins

322   in aflatoxin-contaminated foods, and (ii) hepatitis B (HB) virus for being endemic in the areas with

323   high aflatoxin dietary intake and high incidence of liver cancer [56]. Moreover, by comparing

324   aflatoxin dietary exposure estimates with the incidence of HB virus infections in different USA

325   regions at high-risk of liver cancer, the US Food and Drug Administration (USFDA) strongly

326   supported the view that HB virus was the actual cause of the cancer and the occurrence of

327   aflatoxins in foods was only coincidental [57-58]. However, the method used to evaluate the risk

328   was later criticized for the lack of accuracy in the exposure estimates [59]. Nonetheless, the US

329   issued the first regulation on aflatoxins, as food contaminants, in 1965 with a maximum limit (ML)

330   of 30 ppb (1ppb = 1 µg/L or kg product) for total aflatoxins (sum of AFB1, AFB2, AFG1, and

331   AFG2) in foods; this ML was decreased to 20 ppb in 1969 and maintained until today [60]. In

332   1977, After the characterization of AFM1 as a hazard to consumers, the USFDA established the

333   action level of this mycotoxin in milk to 0.5 ppb.

334   For few decades, the causality between aflatoxins and liver cancer remained a major issue

335   of debate internationally, while research work kept generating supportive data to reach a consensus

336   regarding the carcinogenicity of aflatoxins. By the mid 1970s, the carcinogenicity and

337   mutagenicity of aflatoxins have been extensively demonstrated in different animal species and in

338   bacteria. In addition, many epidemiological studies have inferred a positive correlation between

339   aflatoxin dietary intake and liver cancer in humans. All relevant reports available by 1975, were

340   critically examined by the IARC working group who advised to maintain the previous status

341   considering the outcome of the review as a "circumstantial evidence" for the carcinogenicity of

342   aflatoxins in humans [61]. Although laboratory tests had shown beyond doubt that the

343   administration of aflatoxins via different routes to animals, including birds, ruminants, fish,

344   rodents, and non-human primates caused liver cancer, the IARC working group had many

345   reservations on the rationality of epidemiological and clinical studies. This precluded the group

346   from concluding as to the existence of a direct causal link between aflatoxins and carcinogenicity

347   in humans. One of the major reservations was the fact that most of the cohort and case-control

348   studies had been conducted in developing countries where the highest rate of liver cancer was

349   recorded, which shed doubts on the accuracy of the registration, diagnosis, and completeness of

350   the medical files. In addition, the most significant epidemiological studies have been carried out

351   retrospectively with no certainly that the records and analytical tests were comprehensive, properly

352   conducted, and the records well archived. Moreover, technical flaws were noted regarding the

353   correlation studies, most of which have also omitted to take into account the incidence of chronic

354   hepatitis B and C infections to rule out or confirm the possible interference of these major risk

355   factors with aflatoxins in causing liver cancer [62].

356   The debate continued until 1987 before the IARC finally classified the "naturally occurring

357   mixtures of aflatoxins" in the group 1 carcinogens considering the availability of new tangible data

358   generated from better designed studies [62]. Yet, the specific role of each of HB virus infection

359   and aflatoxins to trigger liver cancer remained to be clarified. By 1992, high-quality studies

360   meeting the IARC provisions to circumvent the limitations raised in previous reviews, have

361   provided "sufficient evidence" for the carcinogenicity of AFB1. Notably, a cohort study has

362   established an almost linear relationship between the dietary intake of AFB1 and the mortality rate

363   from liver cancer, thereby substantiating the direct implication of this particular aflatoxin in the

364   disease [63]. It also unraveled the intricate overlap between the high incidence of HB virus

365   infections and the high dietary exposure to aflatoxins in areas at high-risk of liver cancer by

366   confirming their synergistic effect. The study demonstrated that the risk of liver cancer for

367   individuals with a high aflatoxin intake increased by approximately 13-fold in seropositive

368   individuals for HB-surface antigens (HBsAg) compared with HBsAg-seronegative individuals.

369   Conversely, the mortality rate from liver cancer of HBsAg-seropositive individuals was 10 times

370   lower in areas with low aflatoxin dietary exposure. In addition to the well-designed statistical

371   analysis, the mains strengths of this study were the prospective follow-up of the cohort participants

372   and the use for the first time of biomarkers to assess aflatoxin exposure. The biomarkers used were

373   the AFB1 metabolites AFP1 and AFM1, and the DNA-adduct AFB1-N^7-Gua, whose

374   concentrations were determined periodically in the urine of the cohort members. These results

375   were confirmed by other robust prospective cohort and case-control studies using similar approach

376   and different biomarkers [64-65].

377   In view of the availability of new convincing studies reporting on a direct link between

378   AFB1 dietary exposure and liver cancer, this aflatoxin was included in group 1 carcinogens in the

379   1992 edition of the IARC monographs [66]. However, AFM1 was classified in group 2B of

380   "possibly carcinogenic substances" because of "inadequate evidence" of carcinogenicity in

381   humans; whereas, it was concluded to the "lack of evidence" for carcinogenicity in humans of

382   AFG1, AFG2, and AFB2, and to "inadequate evidence" and "limited evidence" for the

383   carcinogenicity in experimental animals of AFG2 and AFB2 aflatoxins, respectively [66]. These

384   conclusions were reaffirmed by the IARC in 2002, with the provision of additional confirmatory

385   data from a number of well-designed prospective cohort studies supported by laboratory analyses

386   using specific biomarkers of exposure [67]. In 2004, the most deleterious aflatoxicosis ever

387   recorded worldwide occurred in Kenya due to the consumption of highly contaminated maize

388   affecting 317 cases with 125 deaths [68-69]. In addition to the provision of an additional evidence

389   of aflatoxin implication in acute intoxications, this incident revealed a high positive correlation

390   between the levels of aflatoxin-lysine adduct in the serum and AFB1 intake [70]. Since then, this

391   adduct has been largely used as one of the most reliable biomarkers to quantitate chronic exposure

392   to aflatoxins.

393   Mechanistic studies demonstrating that the carcinogenicity of aflatoxins arises from their

394   genotoxic action were also taken into account as sensitive state-of-the art tools that help classify

395   chemical agents in the appropriate carcinogenicity group. The first of such studies was conducted

396   by Bressac, Kew, Wands [71] who demonstrated that the carcinogenicity of AFB1 relates primarily

397   to its genotoxicity. According to these authors, AFB1 targets the tumor-suppressor gene *TP53*,

398   also known as *p53*, where it induces a point mutation by substituting the third base G of the codon

399   249 for the base T (AGG to AGT), with a consequent substitution of the amino acid serine for

400   arginine in the gene product (p53-R249S). This mutation arises from the formation of a DNA

401   adduct, 8,9-dihydro-8-(N^7-guanosinyl)-9-hydroxy (AFB1–N^7-guo), via an active intermediate

402   metabolite, aflatoxin B-exo-8-9-epoxide (AFBO), generated in the liver from AFB1 by action of

403   a cytochrome P450 enzyme (CYP450) [64,72-73]. As this appeared to be a general mechanism

404   among aflatoxins, AFB1, AFB2, AFG1, AFG2 and AFM1 were all classified by the IARC in group

405   1 carcinogens in 2012 [74]. Nonetheless, despite the well-established synergistic action between

406   aflatoxins and HB virus to increase the risk of liver cancer, as provided by cohort studies and

407   biomarker analysis, this synergy awaits to be mechanistically clarified [65,75-78]. The use of

408   biomarkers, seems to be a promising means to settle definitely the long-standing debate around

409   the specific role of each of the two major risk-factors, aflatoxins and HB virus infection, in HCC

410   induction [79-80]. Hepatitis virus C (HC) and the blue-green algal hepatotoxic peptides,

411   mycrocystins, which were evoked as additional risk factors that would act in synergy with

412   aflatoxins to cause liver cancer should also be given due attention [62,81].

413   Beyond the substantiation of exposure to aflatoxins, and their mode of action at the

414   molecular level, mechanistic studies are expected to help progressing the ongoing efforts aiming

415   to reduce the incidence of aflatoxins in foods and feeds in order to detect liver cancer at early

416   stages while the prevention or prognostic improvement are still possible [80]. However, selection

417   for the most appropriate and stable biomarkers for specific purposes, and the design of easy and

418   sensitive methods for their detection in secretion fluids, the blood and/or specific organs, remain

419   a challenging issue for future studies [82]. This trend announces the emergence of a new era in

420   mycotoxin research based on advanced molecular biology and nanotechnology techniques [83].

421   The main historical milestones discussed above that have led to our present knowledge on

422   aflatoxins, their publication dates, and research circumstances are summarized in Table 2.

423
424

Table 2: Major milestones in the historical and scientific progress leading to the contemporary knowledge on aflatoxins (up-to 2012).

Year	Study	Action/outcome	References
1960	Outbreak of turkey "X" disease in the Eastern and Southern regions of London (England) poultry farms	Starting a wide investigation to understand the disease	[2]
1961	Association of the X disease to the imported Brazilian groundnut meal	Removal of Brazilian groundnut from rations and reduction of the disease incidence	[2]
	In vivo confirmation of the toxicity of Brazilian groundnut to other birds and animals	Characterization of typical symptoms and organ lesions caused by the disease	[2,18-23]
	Preparation of a concentrate of the toxic principle in an aqueous suspension	Development of reliable quantitative biological toxicity assay	[22]
	Association of *Aspergillus flavus* contamination with the toxicity of groundnut	Establishing the relation between toxic fractions from cultures of *A. flavus* and fluorescence under UV light	[18]
1962	Improving separation and purification techniques of *A. flavus* culture extracts by thin-layer chromatography (TLC)	New separation methods for aflatoxin purification Identification of aflatoxins B and G	[30]
	Developing crystallization methods	Preparation of crystalline aflatoxins	[29,31]
	Studies on physicochemical properties of aflatoxins	Isolation and chemical characterization of AFB1	[32]
1962	Animal trials for the fate of aflatoxin after ingestion of contaminated feed	Detection of a toxic principle, "milk toxin", in milk drawn from cows fed on toxic groundnut	[36]
1963	Purification and crystallization of aflatoxins	Identification and chemical characterization of AFB1, AFB2, AFG1 and AFG2 First chemical synthesis of AFG1 and AFG2	[33]
	Structural studies of aflatoxins B and G	Elucidation of the chemical structures of aflatoxins B and G as difuranocoumarin derivatives	[34-35]
1964	Purification and toxicity testing of the "milk toxin" in dry milk	Partial characterization of the "milk toxin" and its relatedness to AFB1	[40]
1965	Setting safety standard by the USFDA	First regulation of total aflatoxins (ML of 30 ppb in foods) in the US	[60]
1966	Sheep fed on a mixture of aflatoxins to determine organs and/or body fluids secretions where the "milk toxin" is accumulated or secreted	Detection of "milk toxin" in the milk, urine, kidney, and liver Designation of the "milk toxin" as "aflatoxin M"	[38]
	Purification and fractionation of AFM from sheep urine	Separation and physicochemical characterization of AFM1 and AFM2 as hydroxylated metabolites of AFB1 and AFB2, respectively	[39]
1966	Feeding malnourished African children with meals supplemented with peanut as part of an FAO initiative to control kwashiorkor	Liver damage in most children having been beneficiary participants in the initiative	[51]
1967	Studies on *in vitro* chemical characterization of aflatoxins	Complete chemical synthesis of aflatoxin B1	[84]
1969	Revising standard levels by the US FDA	Action level for total aflatoxins in foods lowered from 30 ppb to 20 ppb	[60]
1970	A case-control study on liver failure leading to the death of a teenager fed on mouldy cassava in Uganda	Circumstantial evidence of the implication of aflatoxins in acute intoxication	[54]

425

426   **Table 2:** *Cont.*

Year	Study	Action/outcome	References
1971	First review by the IARC working group of the available studies on the possible relationship between aflatoxin intake and liver cancer	Studies reviewed were considered to provide a circumstantial evidence for the carcinogenicity of aflatoxins in humans	[56]
1975	Second review by the IARC working group of the previous and newly generated data on the causality between aflatoxins and liver cancer	Confirmation of the previous status of "circumstantial evidence" for carcinogenicity in humans	[61]
1977	Regulatory action guidelines of the US FDA	Action level of 0.5 ppb for aflatoxin M1 in milk	[85]
1979	Building evidence for a link between liver damage and aflatoxin intake	The FAO establishes the first provisional acceptable limit of 30 mg aflatoxin per kg meal	[53]
1987	Third review of new available data generated from better-designed studies to address previous recommendations of the IARC working group	Classification of naturally occurring mixtures of aflatoxins in group 1 carcinogens	[62]
1991	Development of mechanistic studies to demonstrate the carcinogenicity of aflatoxins at the molecular level	Demonstration of the genotoxicity of aflatoxin by induction of point mutation in codon 249 of *TP53* tumor suppressor gene	[71]
1992	High-quality design of epidemiological and mechanistic studies on the carcinogenicity of aflatoxins	Establishment of an almost linear relationship between AFB1 intake and liver cancer. Demonstration of synergistic action between dietary intake of aflatoxins and hepatitis virus B hepatocellular carcinoma. First use of AFP1, AFM1, and DNA-adduct in urine as a biomarker for the exposure assessment	[63]
	Review of the newly generated data by the IARC working group	Addition of AFB1 to the group 1 carcinogens	[66]
1997	Cohort studies taking into account available biomarkers to confirm the carcinogenicity of AFB1 by	Recommendation of wider use of biomarkers as reliable tools to assess exposure to aflatoxins and for aflatoxicosis diagnostic	[64-65]
2002	Review of new and previous data on the carcinogenicity of aflatoxins by the IARC working group	Confirmation of the previous status of aflatoxins	[67]
2004	Investigations on a large aflatoxicosis that occurred in Kenya	The first use of aflatoxin-albumin (AFL-lysine) adduct in blood serum as a biomarker for aflatoxin exposure	[70]
2012	Review of previous and new data on aflatoxin carcinogenicity with an emphasis on mechanistic studies on the genotoxicity of aflatoxins and biomarkers	Revision of the previous classification to consider aflatoxins, implicitly including AFB1, AFB2, AFG1, AFG2 and AFM1, in group 1 carcinogens*	[74]

427   *In 2012, the IARC classified aflatoxins in group 1 carcinogens without specifying "mixtures of naturally occurring
428   aflatoxins" and aflatoxin B1 as in the previous versions of the IARC monographs, thereby implicitly including the
429   major aflatoxins (B1, G1, B2, G2, and M1) in this group on the basis of strong evidence for their genotoxicity
430   involving the formation of DNA adducts causing point mutations in the *TP53* gene [74].

431 **3   Challenges and prospects for aflatoxin research**

432   The recent advances in scientific research on toxicology coupled to the increased accuracy

433 and sensitivity of the analytical tools helped improve our understanding on aflatoxins. However,

434 the same advances raised new challenges that the international community should overcome to

435 reach the ultimate goal of reducing as much as possible the incidence of aflatoxins in foods and

436 feeds, and the consequent health risks they pose to humans and animals. Difficulties to meet this

437 goal internationally are greater with the huge economic and technological gaps between

438 industrialized and developing countries, which also represent major barriers to trading in the scope

439 of the open borders advocated by the world trade organization (WTO). These challenges can be

440 technical, ecological, socio-economic, or legislative in nature.

441 *3.1   Technical challenges*

442   As is the case for any hazard of concern to food safety, a minimum of scientific information

443 on aflatoxins is necessary for risk assessment studies aiming to help stakeholders adequately

444 manage the risk. It also helps national, regional, or international regulatory authorities issue

445 science-based legislative provisions to reduce mis/biased interpretation disputes. Such information

446 includes, but not limited to, knowledge of chemical and toxicological properties, biosynthesis and

447 biodegradation pathways, the producing moulds and their ecological niches, and a thorough

448 estimation of the concentrations and the distribution in foods and feeds in a country, a region, and

449 worldwide. The availability of reliable, sensitive, and specific analytical methods is a central

450 element for the generation the necessary data in all the above-mentioned disciplines. Although

451 aflatoxins are the mycotoxins that have received the most attention in this regard and tremendous

452 amount of data has built up since their discovery, important gaps remain to be filled through further

453 efforts on technical issues.

454   *3.1.1*   Analytical methods and challenges for future development

455   There are many accurate, specific, and sensitive chromatography- and immunology-based

456   techniques routinely used in scientific research on aflatoxins or in regulatory laboratories for

457   official control purposes. Novel methods, such as those using biosensors and optical-based systems

458   intended for rapid in-field and laboratory use to quantitate, semi-quantitate, or screen for aflatoxins

459   are also being increasingly used awaiting improvements in their accuracy and sensitivity to be

460   fully validated [4,86]. The conventional chromatography- and immunology-based methods are the

461   most reliable and widely used but they are costly, time-consuming, or require skilled personnel to

462   be operated [4]. Therefore, they are not readily accessible to developing countries where the

463   problems of aflatoxins are the most severe [87-88]. Due to the global concern of aflatoxins, which

464   can possibly cross borders via international trade, it is urgent to develop easy-to-use, low-cost, and

465   yet reliable methods to meet the needs of smallholder farmers, and small-and-medium enterprises

466   (SME) of food and feed processors in developing countries and elsewhere. They can afford to

467   monitor the levels of aflatoxin-contamination of their produce and take corrective actions to reduce

468   the contamination when necessary. At the same time, this will generate data for meaningful

469   determinations of exposure as a key element for accurate risk assessment, an approach necessary

470   to issue science-based food safety standards. These techniques can also be used in official

471   laboratories to perform efficient and cost-effective controls for proper enforcement of national

472   regulations. Rapid methods of emerging analytical technologies using kits or portable devices such

473   as biosensors and optical-based systems appear to be the best candidates to fulfill such a goal, but

474   they require further refinements to be validated for quantitative analysis [4,89-91]. Although the

475   accessibility to analytical methods cannot guarantee, by itself, efficient reduction of aflatoxin

476   contamination that requires laborious efforts at different levels, it is a prerequisite to any action to

477   be successfully undertaken. It also provides an important yardstick for producers to monitor

478   aflatoxin levels and appraise the efficacy of the quality assurance approach they may adopt to

479   mitigate the incidence of aflatoxins. Only such dynamic can permit gradual reduction in aflatoxin

480   contamination in the most affected countries to support periodic updates of the national regulations

481   in the perspective of global harmonization of food safety standards advocated by the sanitary and

482   phytosanitary (SPS) agreement [92-93].

483   Beyond the level of development of a country or a region, recent discoveries of emerging

484   mycotoxins or those that had been chemically modified during processing or as a result of

485   microbial, animal, or plant metabolism (conjugated or masked mycotoxins) raise new challenges

486   in terms of detection and quantification [94-95]. Moreover, the number of mycotoxin-derived

487   metabolites and mycotoxin precursors that continues to grow with the advance of metabolomic

488   studies adds to these challenges [91]. Apart from the putative or confirmed toxicity of these

489   compounds, they may act in synergy to increase toxicity of the classical mycotoxins, including

490   aflatoxins. Co-occurrence of emerging and modified/masked mycotoxins with regulated

491   mycotoxins is a common phenomenon in agricultural commodities; and a typical commodity

492   contains 7 to 75 different mycotoxins, with an average of 30 [94,96-97]. Failure to detect all

493   mycotoxins and their metabolites in food and feed may result in misdiagnosing mycotoxicoses or

494   underestimating the associated health risks [97].

495   From the about 18 known types of aflatoxins, only AFB1, AFB2, AFG1, AFG2, and AFM1

496   are presently regulated in some commodities in a number of countries; and they are analyzed by

497   conventional techniques. The other aflatoxins are overlooked from the regulatory standpoint

498   despite their potential toxicity or ability to convert into their toxic parents. For example, aflatoxicol

499   that contaminates milk and dairy products has the same toxicity as aflatoxin M1 and is readily

500 converted into its highly toxic parent aflatoxin B1 in the liver [98-99]. Although parasiticol is

501 weakly mutagenic and probably non-carcinogenic, it has the same acute toxicity as B1 and may

502 be of concern to food safety [100]. Similarly, many aflatoxin precursors, such as those of AFB1,

503 were shown to be toxic to different extents [101]. Yet, these toxins are not subject to routine

504 analysis in foods and feeds, nor are they detected by the conventional methods. Furthermore,

505 despite the lack of data on the interactions (antagonism or synergy) of the emerging mycotoxins

506 with aflatoxins, their detection and quantification remain crucial to have a clear picture of such

507 interactions and for accuracy of risk assessment studies.

508   In view of the above considerations, intensive work has been done during the last decade

509 to develop methods capable of detecting multiple analytes of different nature (e.g. mycotoxins and

510 their metabolites, pesticides, fungicides, and veterinary drug residues, and plant toxins) in one

511 sample analysis. Many state-of-the-art technologies are being considered to develop multi-

512 mycotoxin analysis with simplified sample preparation and a validated "fit-for-purpose" status

513 [95]. Development of such multi-analyte/multiplex techniques based on liquid chromatography

514 (LC) coupled with mass-spectrometry (MS) or with high resolution mass-spectrometry (HRMS)

515 received increased attention to address the challenge. The first validated of such techniques was

516 LC coupled with a tandem MS (LC-MS/MS), which could simultaneously analyze quantitatively

517 39 different mycotoxins, including emerging and masked mycotoxins, as well as derived-

518 metabolites in maize and wheat [102]. Based on this technology, Biomin Holding (GmbH, Austria;

519 https://www.nationalhogfarmer.com) developed multi-mycotoxin analytical devices, e.g.,

520 Spectrum 380®, which can analyze simultaneously more than 450 different fungal metabolites,

521 encompassing all of the known mycotoxins and their derived metabolites [91]. A fit-for-purpose,

522 cost-effective LC-MS/MS multi-mycotoxin method, validated for 13 different mycotoxins,

523   including aflatoxin B1, was recently applied to survey the multi-mycotoxin occurrence in maize

524   and wheat produced in South Africa [103]. Computer-assisted techniques using LC-HRSM was

525   suggested to be the most promising LC-MS-based technology; it has the same performances as the

526   LC-MS/MS with an additional advantage of allowing retrospective analysis to screen for

527   mycotoxins that had not been regulated at the time of sample analysis [60]. In addition, this

528   technique can be fit to metabolomic studies for the detection of thousands of low molecular-weight

529   metabolites in a wide range of concentrations in a single analysis [91]. The emerging "omic"

530   discipline of metabolomics is, therefore, expected to reveal novel secondary metabolites of moulds

531   not yet known, thereby extending the repertoire of mycotoxins to include non-targeted novel

532   mould metabolites that can be either toxic or detoxified derivatives. This is achieved by the stable

533   isotype labeling (SIL) technique whereby a biological system, e.g., a plant, is treated with a native

534   mycotoxin and its uniformly labeled ^{13}C counterpart (1:1). The fate of the precursor is then traced

535   by LC-HRSM to generate data and processe them by an appropriate software [104]. Applied to

536   wheat artificially inoculated with deoxynivalenol (DON), this technique revealed the presence of

537   8 novel DON derivatives, suggested to have resulted from a detoxification strategy of the plant

538   [105]. Although no such studies, to our knowledge, have been done on aflatoxins they are strongly

539   encouraged to provide a comprehensive view on the constitutive members of this important group

540   of natural toxicants and its biological detoxification. Indeed, metabolomics can also be fit for gene-

541   function studies to relate, for example, the genetic inheritance of the ability of an organism to

542   metabolize/detoxify a mycotoxin. This can in turn be useful for the development of

543   decontamination strategies of foods and feeds.

544   Despite the high performances of the above discussed techniques and the undeniable

545   potential they have, as avant-garde technologies, to advance analytical science, their high cost

546   (equipment and reagents) and their requirement for highly trained personnel limit their

547   affordability by developing countries as is currently the case of conventional techniques. In

548   addition, they are facing technical limitations mostly related to the management of matrix effects,

549   such as:

550   -   The need to perform tedious multiple clean-up steps prior to the analysis for detection,

551   identification, and quantification, depending on the intended use of the analysis,

552   -   The need for calibration to ensure that the concentrations of all mycotoxins in a sample fall

553   within their respective ranges (i.e., linearity of the calibration curve for all mycotoxins to be

554   determined). A procedure that can be laborious given the wide variations in concentration

555   ranges of the mycotoxins in a sample (ng/ml for some mycotoxins vs μg/ml for others). The

556   extract should then be concentrated or diluted of as appropriate, usually leading to

557   compromises at the expense of the sensitivity. Uniformly labeled mycotoxins for internal

558   standards have been successfully used to circumvent this limitation, but this was faced the

559   availability and affordability. Limited availability and high cost of reference standards for

560   external and internal calibration are especially crucial impediments for detection and

561   quantification of emerging and modified mycotoxins,

562   -   Cross-talk across assays resulting from the interference between signals of different

563   mycotoxins in the same sample may hinder mycotoxins with weak signals, leading to falls

564   negative results.

565   These limitations and the strategies to cope with them have been thoroughly reviewed

566   previously [60,91,95,106-107], and there is a general agreement on the absence of a universal

567   solution to eliminate the matrix-effect in all cases. Therefore, this aspect represents one of the

568   major challenges that researchers will be facing in the coming years to increase the sensitivity,

569   accuracy, repeatability, and efficacy of the LC-MS-based techniques for multi-mycotoxin analyses

570   with a minimal sample preparation. It was suggested that overcoming these limitations may

571   revolutionize the techniques to make them fully automated with a minimum human errors [60].

572   Multi-mycotoxin analysis using multiplex assay kits such as those described for drug development

573   may also be a feasible and affordable solution, but they also need improvements for the accuracy,

574   sensitivity, and specificity to be validated for intended purposes [108].

575   *3.1.2*   Dosimetry of aflatoxins and risk prediction with biomarkers

576   The science-based provisions of the SPS agreement enforced in 1995 emphasize the central

577   role that risk assessment should play in food safety regulations (Article 2.2) to promote the global

578   trade while maintaining an appropriate level of protection (ALOP) of health and life [93,109].

579   Consequently, research on the dietary intake of hazards has been accelerated for quantitative

580   determination of health risks as a basis to determine the tolerable daily intake (TDI) and thereby

581   set the maximum limit of contaminants in foods and feeds. Aflatoxins were among the first

582   chemical hazards whose health risk was assessed and the outcome used to issue or revise regulatory

583   standards in different countries as well as in the *codex alimentarius* (CA)*,* the benchmark for

584   international trade. However, the accuracy and completeness of the published data remain

585   hampered by the high uncertainties and inconsistencies in the estimations of food consumption

586   and the levels of food contamination with aflatoxins; the two crucial parameters for dietary

587   exposure estimation [88]. This holds especially true for developing countries where the diet

588   consists mainly of self-produced and traditionally made foods of doubtful sanitary quality and that

589   are usually neither declared nor controlled for sanitary quality before consumption [88]. Besides,

590   most of the good quality foods produced in these countries are exported while those that fail to

591   meet the safety standards and those that are not controlled are marketed locally via unformal routes,

592   thereby increasing uncertainties with a tendency to underestimate the exposure. An alternative

593   epidemiological approach using biomarkers in biological fluids, mainly blood and urine, has been

594   developed and is gaining increased interest [88]. The main aim of this approach is to develop and

595   validate biomarkers for quantitative estimation of exposure to aflatoxins and associated health

596   risks. Biomarkers can also be useful to predict the risk of disease/cancer development ahead of the

597   onset to allow the implementation of preventive measures such as chemoprotective strategies or

598   diet change [110]. Table 3 summarizes the main biomarkers presently known with their limitations

599   and strengths.

600   Currently, aflatoxin-albumin adduct in blood serum is the most reliable biomarker for a

601   long-term exposure to AFB1 owing to its longest half-life (20 days on average) compared with

602   any other known urinary adduct, and it persists in the serum for more than 3 months [82,111]. A

603   highly positive correlation between aflatoxin dietary intake and the level of aflatoxin-albumin

604   adduct in human serum has been repeatedly demonstrated in humans and animals; and a percentage

605   of 1.4 to 2.3 of ingested aflatoxin was shown to bind covalently serum albumin [112-113]. A series

606   of studies using this adduct as a biomarker demonstrated a strong dose-response relationship

607   between aflatoxin exposure *in utero* or during early infancy and growth impairment [114].

608   Nonetheless, the significance of this biomarker as a molecular dosimeter for quantitative risk

609   assessment or as a predictive parameter to identify individuals prone to develop HCC was

610   questioned [115]. The controversy arises from the fact that the metabolism of AFB1 via the

611   genotoxic epoxide formation pathway is not the only source for the generation of aflatoxin-

612   albumin adduct [116]. In the genotoxic pathway (Figures 1 and 2), this adduct derives from the

613   metabolism of AFB1 in the liver where it is first oxidized by cytochrome P450 enzymes (CYP3A4,

614   CYP1A2, and CYP3A7) to form AFB1-exo-8,9-epoxide (the highly reactive genotoxic metabolite)

615   and AFB1-endo-8,9-epoxide (less active). These unstable epoxides are spontaneously transformed

616   into AFB1-8,9-dihydrodiol which undergoes a base-catalyzed ring opening to aflatoxin-

617   dialdehyde that in turn reacts by Schiff base formation with the lysine side chain of serum albumin

618   to form the AFB1-lysine adduct. This pathway leads to the formation of the adduct that can

619   adequately inform on AFB1 exposure and its potential to induce HCC. However, aflatoxin-

620   albumin adduct was recently suggested to also derive via an alternative pathway from $AFB2_a$ [116].

621   Accordingly, $AFB2_a$, from the diet or as an AFB1 metabolite, is directly oxidized to $AFB2_a$-

622   dialdehyde and reacts by a dual condensation with serum albumin to form $AFB2_a$-albumin adduct

623   with a pyrrole ring, contrary to aflatoxin B1-albumin adduct that has a typical pyrrolin-2-one ring

624   [116]. Under oxidative conditions, the pyrrole ring of the aflatoxin $B2_a$-albumin adduct is oxidized

625   to yield the adduct (with a pyrrolin-2-one ring) as that formed via the genotoxic pathway directly

626   from aflatoxin B1 (Figure 2). In the light of these findings, the authors concluded that aflatoxin-

627   albumin adduct may not always be a reliable indicator of aflatoxin B1 intake and its subsequent

628   metabolism through the genotoxic pathway. This is consistent with an earlier report on the lack of

629   significance of serum albumin-adduct levels for quantitative risk determination and for accurate

630   prediction of the risk for HCC development [115]. The lack of correlation may be further enhanced

631   by the fact that the aflatoxin-dialdehyde does not react entirely with serum albumin and part of it

632   is reduced with aflatoxin aldehyde reductase (AFAR) into aflatoxin-dialcohol, which then reacts

633   with glucuronic acid under the action of a UPD-glucuronosyltransferase to be detoxified in the

634   form of aflatoxin-glucuronide and excreted in the urine (Figure 1). Moreover, part of the $AFB2_a$

635   produced in the liver binds covalently to cellular proteins and phospholipids (Figure 1) instead of

636   binding albumin [116]. These various pathways and fates of precursor and intermediate

637   metabolites make the quantitative correlation between the ingested AFB1 and the resulting

638   aflatoxin-albumin adduct dependent on different factors that drive the metabolic reactions in favor

639   of one or another pathway. Nonetheless, aflatoxin-albumin adduct is currently the most reliable

640   biomarker which was validated to determine aflatoxin "chronic" exposure.

641   Aflatoxin-N^7-guanine is an aflatoxin-DNA adduct that has also been intensively used as a

642   biomarker to estimate recent exposure to aflatoxin and, hence, to diagnose acute aflatoxicosis, or

643   identify individuals or groups of people at high risk of liver cancer [117]. This adduct results from

644   the metabolism of AFB1 in the liver involving different cytochrome P450 enzymes (e.g., CYP3A4,

645   CYP1A2, CYP3A7) to form the highly reactive intermediate metabolite AFBO, which binds

646   covalently to the N^7 of the DNA guanine residue forming the 8,9-dihydroxy-8-(N^7) guanyl-9-

647   hydroxy (AFB1-gua). The AFB1-gua is unstable due to electric charge interactions within the

648   DNA molecule and is, therefore, rapidly released from the DNA leaving an apurinic site. The free

649   adduct is then excreted exclusively in the urine where it serves as an exposure and risk biomarker

650   [118]. Alternatively, it is stabilized on the DNA by opening the ribose ring to form an AFB1-

651   formamidopyrimidine (AFB1-FAPY), which can thus be useful to measure the effective biological

652   dose and, hence, for dose-response characterization and disease outcome. The concentrations of

653   both of the latter aflatoxin-DNA adducts (AFB1-gua and AFB1-FAPY) in tissue samples taken

654   from the liver and kidney of mice were shown to be proportional to the administered levels of

655   AFB1 [119]. A highly positive correlation was also demonstrated in humans between AFB1-gua

656   adduct in the urine and the dietary intake of AFB1 [65,110,117]. However, the major limitation of

657   the latter metabolite as a biomarker resides int that it can only inform on a recent intake of the

658   aflatoxin or be used to monitor exposure changes in individuals subject to interventions, such as

659   chemoprevention treatment or exposure-avoidance through a specific diet [115,117,120]. Despite

660   the evident advantages AFB1-FAPY as a risk biomarker, the accessibility to tissue samples is a

661   major limitation and has, so far, been done only in experimental animals or post-mortem

662   histopathological examinations. Both AFB1-gua and AFB1-FAPY were suggested to be used for

663   histopathological diagnosis to relate the etiology of liver cancer to aflatoxins [121].

664   Aflatoxins B1 and its metabolites excreted in the urine are also routinely used as

665   biomarkers (Figure 1). The so-called phase I metabolites of AFB1, such as AFM1, AFP1, AFQ1,

666   aflatoxicol, and AFB2$_a$ produced in the liver by various cytochrome P450 enzymes (Figure 1) have

667   been considered as exposure biomarkers for internal dose determinations and for possible

668   correlation between their levels and risk of HCC development. The excretion of AFM1 and AFP1

669   was first demonstrated in the urine from mice fed on AFB1-contaminated rations [122]. The study

670   also reported these metabolites (AFM1 and AFP1) were excreted in urine from humans exposed

671   to AFB1 through the diet, and a dose-response relationship between AFM1 and HCC has been

672   established in cohort studies in Taiwan [123]. Also, the amount of AFM1 in the urine of individuals

673   from the aflatoxin-endemic Guangxi region of China was correlated with AFB1 intake, and a

674   proportion of 1.23 to 2.18% of dietary AFB1 was excreted in the urine as AFM1 [124]. The

675   presence of AFP1 in urine samples was directly related to the risk of liver cancer [63], but no linear

676   relationship between its levels in the urine and exposure to AFB1 could be demonstrated [110].

677   Meanwhile AFQ1 was rarely detected in urine samples from people or animals fed on AFB1-

678   contaminated foods or feeds [122].

679   Phase II metabolites, other than DNA and protein adducts discussed above, namely

680   aflatoxin-glucuronide and aflatoxin mercapturic acid derived from aflatoxin-dialcohol and

681   aflatoxin-GSH conjugate, respectively (Figure 1) are additional potential urinary biomarkers that

682   have received a limited attention [80]. However, the usefulness of aflatoxin mercapturic acid as an

683   indicator of the effectiveness of chemoprevention strategies has been emphasized [80,82,120,125].

684   Similarly, aflatoxin-glucuronide would serve the same purpose, but more importantly, it could

685   provide a practical protective means against AFB1 toxicity by the administration of AFAR inducer

686   drugs. This highly inducible enzyme (AFAR) catalyzes aflatoxin-dialdehyde reduction into

687   aflatoxin-dialcohol, which does not form adducts proteins/albumin but is rather detoxified into the

688   non-toxic aflatoxin-glucuronide conjugate [126]. This prevents the formation of aflatoxin-albumin

689   adduct from aflatoxin-dialdehyde and favors the detoxification of AFB1 over the genotoxic

690   pathway. Induction of AFAR by natural or synthetic antioxidants and other drugs was

691   demonstrated in rats and shown to increase their resistance to AFB1 [80,127-128].

692   Other AFB1 metabolites have a potential for use as risk biomarkers and for the biologically

693   effective dose measurements to provide reliable indication on the exposure and a dose-response

694   effect. These are, for example, AFB1-exo-8,9-epoxide and AFB1-FAPY, directly involved in the

695   mechanism of toxicity but since they are not excreted in biological fluids, their levels should be

696   determined in tissues of target organs, which is not always feasible. There is, therefore, a need to

697   develop specific and safe sampling and analytical procedures to take advantage of these

698   intermediate metabolites that would provide valuable and precise information to establish the dose-

699   response effect (i.e., genotoxic dose) necessary for quantitative risk assessment. Other molecular

700   biomarkers in this category may be identified in the future, as metabolic pathways and

701   pharmacokinetics of aflatoxins are being progressively elucidated.

702   According to Groopman and Kensler [110], the validation of a biomarker should undergo

703   a stringent procedure for its suitability to provide pertinent information spanning from exposure to

704   the outcome of a disease and be experimentally tested for sensitivity, specificity, accuracy, and

705   reliability through pilot studies on humans, among other provisions. Under these conditions, few

706   biomarkers meet individually all the required validation criteria. As matter of fact, the challenge

707   for efficient use of biomarkers to assess and predict the risk of cancer development in individuals,

708   communities, and larger-number populations has been raised for over two decades [110] and little

709   progress has been made since then due to limitations of each of the presently known biomarkers

710   (Table 3). The same authors suggested an alternative approach consisting of using composite sets

711   of biomarkers, each of which addresses specific criteria so that whole group of sets meets the

712   overall validation requirements. Therefore, it seems more appropriate for future studies to

713   investigate the combinations of biomarkers that would provide complementary information to have

714   the most accurate and precise indication on the extent of exposure to predict the disease risk and

715   outcome [129-130]. Success of this approach is contingent to the availability of appropriate and

716   easy-to-use analytical techniques that can apply to all biomarkers in a composite set at once.

717   Presently, immunology- and/or chromatography-based techniques are the most used to quantitate

718   biomarkers individually and they should usually be adapted to a specific biomarker under specific

719   conditions to fot-for-purpose. ELISA, radioimmunoassay (RIA), HPLC with fluorescence (HPLC-

720   Fl) detection, LC-MS and LC-MS/MS, LC-HRMS, and atomic mass spectrometry (AMS) are

721   frequently used, each of which bears advantages and limitations [114]. Again, liquid

722   chromatography coupled with isotope dilution tandem mass spectrometry and LC-HRMS were

723   recently shown to be the most accurate, precise, specific, and sensitive [131-132]; yet, the cost and

724   the need for skilled personnel to perform the analyses are their main drawbacks. To this end, the

725   development of multianalyte techniques, discussed above, specifically designed to quantitate as

726   many biomarkers as possible in one analysis, taking into account the matrix effect, warrants

727   consideration. Therefore, the next challenge that research on aflatoxins will be facing, in this

728   particular issue, is to find the most appropriate set of biomarkers to be used together and the most

729   reliable techniques for their detection and quantification.

730   **Table 3:** Biomarkers used to assess exposure or risk posed by aflatoxin B1 in humans, limitations and strengths.

Biomarker	Limitations	Strengths	Detection specimen	Validation status	References
Aflatoxin B1 (parent)*	No correlation with the ingested amount of the aflatoxin	Useful when used along with other biomarkers	Urine and serum	No	[65,133-134]
Phase I metabolites					
Aflatoxicol*	Lack of correlation with aflatoxin intake	May be useful when used along with other biomarkers	Urine	No	[135]
Aflatoxin M1*	Significance for short term exposure only	Major aflatoxin B1 metabolite excreted in the urine. Highly correlated with aflatoxin B1 dietary intake. Evidence for a dose-response relationship with hepatocellular carcinoma (HCC)	Urine	Yes	[65,122-123]
Aflatoxin P1*	Significance for short term exposure only. Lack of correlation with aflatoxin B1 intake	Useful when associated with other biomarkers in providing information on the risk of disease onset and diagnosis	Urine	No	[63,65,117]
Aflatoxin Q1*	Significance short term exposure only. Rarely detected in the urine after exposure to aflatoxin B1	May be useful if used along with other biomarkers	Urine	No	[65,136-137]
Aflatoxin $B2_a$*	Does not necessarily reflect the DNA damaging effect of aflatoxin B1. Lack of correlation with aflatoxin B1 intake	One of the major metabolites of aflatoxin B1 which may inform on acute toxicity (forms adducts with proteins and phospholipids)	Serum, Urine	No	[116,138]
Aflatoxin B1-8,9-dihydrodiol**	Not excreted in biofluids; no easily accessible or available samples	May be a good indicator for acute toxicity, as it leads to the formation of adducts with functional proteins	Liver (*in vitro*)	No	[117]
Aflatoxin B1-exo-8,9-epoxide**	Not excreted in biofluids; no easily accessible or available samples. Short-lived, very unstable intermediate metabolite (difficult to quantitate accurately any time after ingestion)	Directly related to toxicity mechanism; best risk marker for the aflatoxin intake, dose/response determinations, and prediction of the disease onset	Liver (*in vitro*)	No	[72,139-140]
Phase II metabolites (Aflatoxin-Protein adducts)					
Aflatoxin B1-lysine**	Limited value regarding quantitative risk assessment and the prediction of HCC at early stages	Chronic exposure (stable for more than 3 months in serum). Highly correlated with aflatoxin B1 dietary intake. Best used to associate aflatoxin intake and child growth impairment	Blood serum	Yes	[82,113,129,131,141-142]

731

732

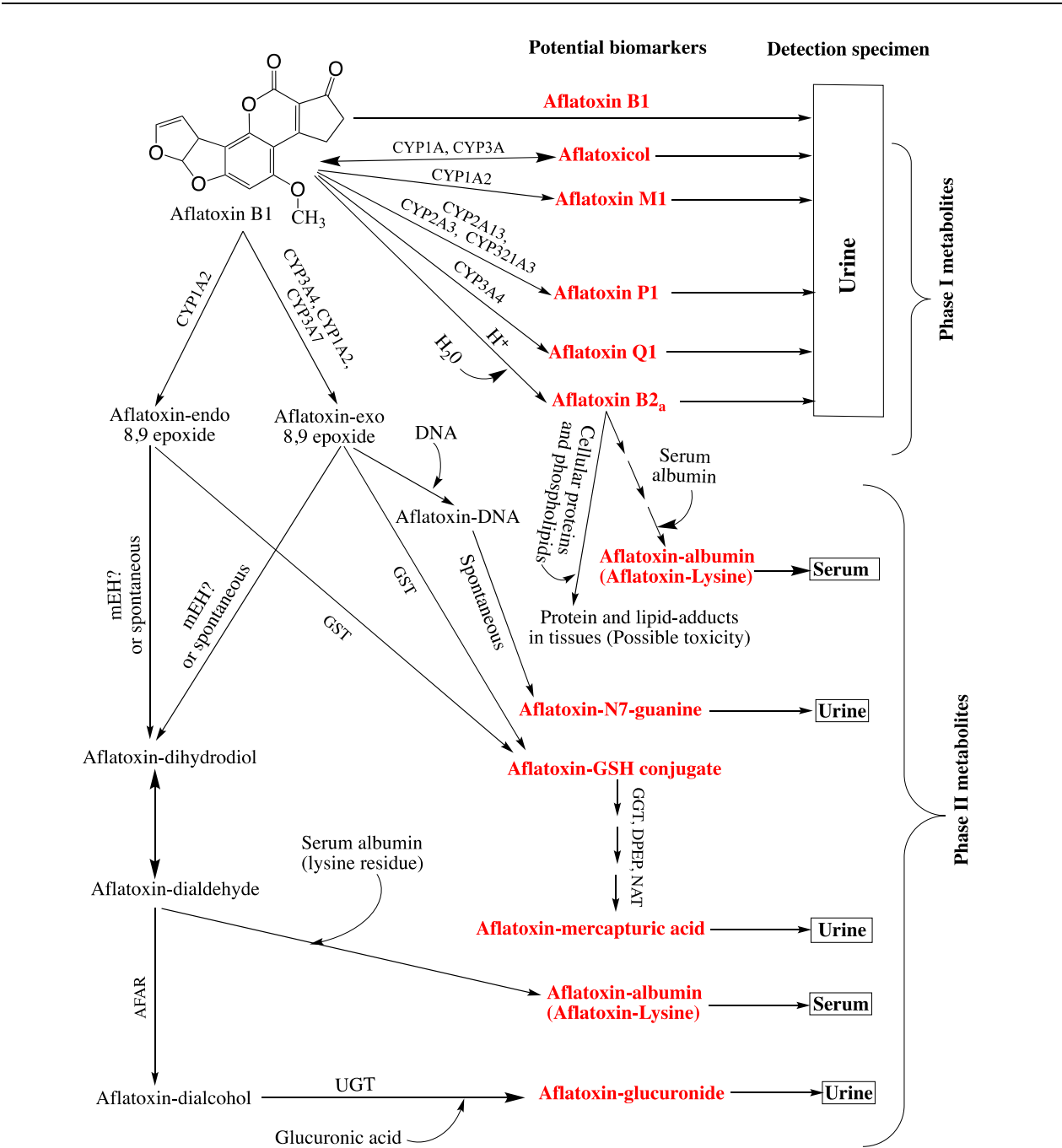
Table 3: *Cont …*

Biomarker	Limitations	Strengths	Detection specimen	Validation status	References
Phase II metabolites (aflatoxin-DNA adducts)					
Aflatoxin B1-N^7-Guanine**	Not valid for a long-term exposure or to predict the onset of liver cancer	Linear proportionality with ingested aflatoxin B1. Confirmation of the etiology of aflatoxins in HCC	Urine	Yes	[122,130]
Aflatoxin B1-FAPY**	Not excreted in biofluids; no easily accessible or available samples	Directly implicated in DNA mutations leading to cancer; suitable marker for dose-response determination and disease outcome	Liver, kidney, viscera (*in vitro*)	No	[119]
Aflatoxin B1-8,9-dihydrodiol**	Idem as Aflatoxin B1-FAPY	May be a good indicator for acute toxicity, as it leads to the formation of adducts with functional proteins	Liver (*in vitro*)	No	[117]
Aflatoxin B-exo-8,9-epoxide**	Idem as AFB1-FAPY Short-lived, very unstable intermediate metabolite	Directly related to toxicity mechanism; best risk marker for the aflatoxin intake, dose/response determinations, and prediction of the disease onset	Liver (*in vitro*)	No	[72,139-140]
Aflatoxin-glutathione	Idem as AFB1-FAPY. Unavailability for sampling; only detected in bile	Best indicator for the detoxification of aflatoxins	Bile	No	[143]
Aflatoxin-mercapturic acid*	Low level in urine does not necessarily indicate a shift towards the genotoxic pathway if the phase I enzymes are inhibited (low levels of epoxides)	Good risk biomarker to monitor the outcome of chemoprotective actions; reflects the induction of the detoxification pathway via AFB1–GSH conjugate formation (phase II metabolites activation)	Urine	No	[120,125,144]
Aflatoxin-glucuronide	Insufficiently studied	Indicator of the of aflatoxin aldehyde reductase (AFAR) activity; potential use to monitor chemoprotection action by administering AFAR inducers	Urine	No	[80]

733
734 * Biomarkers for determinations of the "internal dose", as a measure of the products that are produced in the body from the metabolism of the parent aflatoxin to serve as exposure biomarkers.

735 ** Biomarkers for determinations of the "biologically effective dose", i.e., "*the fraction of xenobiotic capable of interacting with*
736 *cellular macromolecules at the target site. Macromolecular adducts should not only be considered as exposure indicators; indeed,*
737 *their biological significance can also be extended to biomarkers of effect and of susceptibility*" [145].

738

739

740 **Figure 1:** Fate of aflatoxin B1 in the liver showing the metabolites with potential to be used as
741 biomarkers "in red boldface letters" for exposure determination and the biofluids where they can
742 be detected and quantified. For the details on the formation of aflatoxin-albumin adduct from
743 aflatoxin B2a, see Figure 2 below. Adapted from [146]
744 **Abbreviations**: GGT: γ-glutamyltranspeptidase; DPEP: Dipeptidase; NAT: Nacetyltransferase;
745 UGT: UPD-glucuronosyltransferases, mEH: Microsomal epoxide hydrolase; GST: Glutathione-S
746 transferase; AFAR: Aflatoxin aldehyde reductase.
747

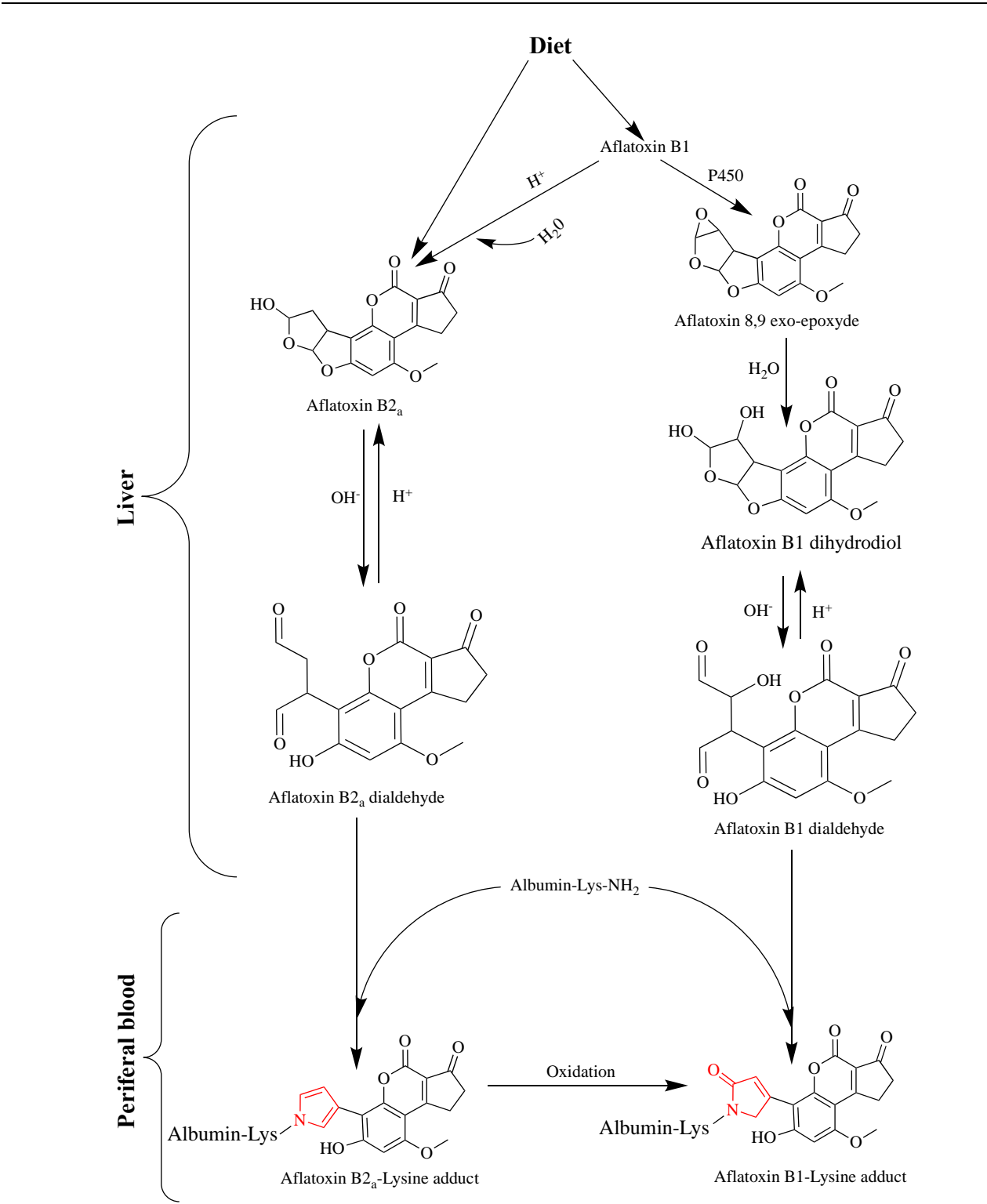



Figure 2: Comparative formation pathways of aflatoxin B1-Lys (lysine) adduct deriving exclusively from aflatoxin B1 (Right) or from aflatoxin B1 and B2$_a$ (Left) as precursors. The pyrrole and pyrrolin-2-one rings that characterize aflatoxin B2a- and aflatoxin B1-albumin adducts are drawn in red. Adapted from [116]

753　*3.2　Natural and socioeconomic challenges*

754　　　　Aflatoxin contamination of foods and feeds is unevenly distributed throughout the world

755　depending on the geographical zones and the prevailing climatic conditions. Countries in the

756　tropical and sub-tropical zones with hot and humid climates located between 40° North and 40°

757　South of the equator are the most exposed to the growth of aflatoxigenic fungi and aflatoxin

758　production in agricultural products [147]. Developing countries in this region, especially those of

759　Southeast Asia and South Africa, have been known for the highest incidence of aflatoxins and

760　rates of related HCC worldwide [1,148]. Sustained efforts involving international cooperation have

761　been made to alleviate the health and economic burden associated with aflatoxins in these countries

762　[149]. However, the efforts have been hampered, in part, by the favorable climates in these

763　countries to the growth of aflatoxigenic molds and aflatoxin production, and which become ideal

764　with occasional drought followed by flooding episodes [69-70]. Under these conditions, aflatoxin

765　contamination of agricultural products can only be controlled by modern high-tech agricultural

766　practices using integrated approach from pre-harvest to consumption. Such an approach is not

767　affordable by farmers of the region, most of whom are smallholders practicing subsistence

768　agriculture with traditional management systems [150-151]. The lack of awareness about the

769　impact of aflatoxins on crop yield and safety from the producers and consumers alike is another

770　hurdle that has been attributed to the poor risk communication and the absence of functional

771　bridges between academic and development institutions [152].

772　　　　Sophisticated weather monitoring systems and the development of reliable predictive

773　models are increasingly necessary to forecast unusual climate events and seasonal variations ahead

774　of their occurrence to take preventive measures when possible [153]. This situation is expected to

775　worsen in the future with the ongoing climatic change and its impact on the extent and emergence

776   of new and/or modified food safety problems, including mycotoxicosis, worldwide. Using

777   predictive modeling to measure aflatoxin risk index (AFI), defined as the potential of *A. flavus* to

778   interact with crop and produce aflatoxin, the mean AFI associated with AFB1-contaminated maize

779   in Europe was estimated to increase during the next 100 years with global warming scenarios of

780   2°C and 5°C by 92% (AFI = 73.25) and 140% (AFI = 95.09), respectively compared with the

781   present status (AFI = 38.20) [153]. According to the study, maize cultivation and its contamination

782   with aflatoxins will expend throughout Europe to reach currently spared high altitudes zones

783   between 45° and 60° North of the equator, while Southern Europe countries are expected to be at

784   high risk with levels of aflatoxins exceeding the legal limits more frequently than is currently the

785   case. This is of more concern in tropical and sub-tropical countries, already facing serious problems

786   of aflatoxin contaminations, with the El Niño-Southern Oscillation (ENSO) phenomenon

787   associated with interannual extreme shifts in rainfall and temperature causing alternating drought

788   and excess rainy periods. As a result, ecological conditions are modified to favor the growth of

789   microbial pathogens with a consequent increase and emergence of foodborne diseases [154]. The

790   increase in food and feed contamination with aflatoxins and the occurrence of aflatoxicosis

791   outbreaks subsequent to severe drought followed by unseasonal heavy rains is well documented

792   [70,147,155-157]. Climatic change was suggested to affect the expression of the regulatory genes

793   *AflR* and *AflS*, as well as the early structural gene *AflD* in *A. flavus* and *A. parasiticus*. The

794   expression of these genes is significantly influenced by ecological stress factors, mainly

795   temperature and water availability; highest production of AFB1 was demonstrated to occur at a

796   temperature/a_w combinations of 25°C/0.95, 25°C/0.99, 30°C/0.95, and 30°C/0.99 [158]. With the

797   ongoing climatic change, such environmental conditions for aflatoxin production are likely to be

798   common in many regions throughout the world. During the dry period, the temperature exceeds

799   25°C, and the excess rain that usually follows increases the water activity to above 0.95 providing

800   ideal conditions for aflatoxin production. Unless, robust measures with modern high-tech

801   management systems are taken, crop contamination with aflatoxins will undeniably tend to

802   increase. This strongly suggests, that in additions to control means for aflatoxin contamination at

803   the production, in-farm storage, and market levels, there is a need to curb the climatic change as

804   the driving factor. Therefore, one of the most challenging issues that scientific

805   research/development on aflatoxins will be facing for many years to come is probably the impact

806   of climatic change on aflatoxin contamination of foods and feeds. It is a multifaceted issue that

807   requires immediate and anticipatory actions with high degree of coordination between

808   education/research and development institutions, international cooperation, various organs of the

809   United Nations, non-government associations, etc. Appropriate monitoring of climatic change

810   with sophisticated analytical and modeling tools with the provisions of regulatory measures to

811   prevent environment pollution or allow its rehabilitation is necessary and involves joint

812   international efforts. Moreover, research and development programs should use innovative ways

813   of partnership between scientists, developers, and funding institutions for the highest possible

814   impact of the results in the field. In particular, the involvement of social scientists as key players

815   in any research project on aflatoxins have been suggested to be an imperious need to bridge

816   scientific findings and the reality of the social context [152].

817   *3.3   Actions to mitigate aflatoxin-induced health risks*

818   The ultimate goal of scientific research on aflatoxins is the development of efficient means

819   to alleviate the health risk they pose to humans and animals. Despite the tremendous efforts that

820   have been done to reduce the incidence of these natural toxicants in foods and feeds since their

821   discovery, they are still widely distributed at high levels in nature and continue to raise serious

822   public health concerns. They are also one of the main barriers to international trade of agricultural

823   products, especially between developing and industrialized countries. Actions to improve the

824   situation can be undertaken at two main levels: (i) at the food and feed level by reducing their

825   contamination and hence the dietary exposure, and (ii) at the consumer level by adopting strategies

826   to prevent or retard the onset of the diseases, especially the HCC, in populations or individuals at

827   high risk [80].

828   *3.3.1   At commodities' level: Reducing the dietary intake*

829   It is well established that safe decontamination of foods and feeds from aflatoxins is a real

830   challenge, and that only preventive integrated holistic approaches involving quality assurance

831   systems can yield satisfactory results in reducing the risk associated with aflatoxins. Actions

832   should be undertaken at all the production stages; from pre-harvest to post-harvest, including

833   irrigation systems, selection of resistant cultivars (natural selection or genetically modified crops),

834   pest control, monitoring climatic conditions, managing the time of harvest, drying techniques,

835   transportation, conditioning, and storage (in-site, in the market, and at home) [159-160]. While

836   this approach can reasonably be followed in industrialized countries, it is not affordable for

837   developing ones where agriculture and livestock production are mainly practiced for subsistence

838   by low-income smallholder farmers. [161] Similar situation applies to the food and feed industries

839   predominated by SMEs and SMIs with insufficient capacity to afford high technology

840   requirements. To avoid losses caused by disposal of agricultural products deemed to be unfit for

841   consumption for their high aflatoxin content, numerous detoxification/decontamination techniques

842   using biological, chemical, and physical treatments have been designed to ensure "safe" levels of

843   contamination before consumption [160,162-166]. Depending on the product, some of these

844   treatments prove efficient in removing or detoxifying aflatoxins from/in certain foods and feeds

845   [164]. Yet, to find practical applications, a treatment should possess other features, including low

846   cost, easy to use, no partial toxicity retention or generation of new toxic compounds, and no

847   adverse effects on the nutritional or sensory quality of the product. These characteristics are rarely

848   met in a single treatment, which can, in addition, apply to all commodities and for all known

849   aflatoxins. On the other hand, the impact of these strategies at the global level remains far below

850   expectations due to the wide technological and economic gaps between developing and

851   industrialized countries. Beside the ongoing research/development programs on

852   detoxification/decontamination means of foods and feeds, additional efforts should be made to

853   foster international cooperation in order to allow developing countries implement quality

854   assurance programs and good agricultural practices. Nevertheless, this essential prerequisite to

855   make a real progress in this issue globally can only be achieved on the long run and depends largely

856   on the implementation of adequate politics to promote international development.

857   *3.3.2   At consumers' level: Chemoprotection/chemoprevention*

858   Under certain conditions, aflatoxin contamination cannot be significantly restricted in

859   foods and feeds most of which remain highly contaminated while their condemnation would

860   seriously compromise food security. This is true for most developing countries where some extent

861   of unsafe levels of aflatoxin contamination must be accepted providing intervention strategies are

862   applied to interfere with the toxicity or limit the bioavailability of aflatoxins.

863   *3.3.2.1   Reducing the risk by interfering with the toxicity of aflatoxins*

864   To express their toxicity in humans or animals, aflatoxins should first be activated to form

865   reactive aflatoxin-exo- and -endo-epoxides by the action of mono-oxygenase cytochrome enzyme

866   systems (CYP P450). These products, especially the highly reactive aflatoxin-8,9-exo-epoxide,

867   bind nucleic acids (RNA, DNA) forming adducts, predominantly aflatoxin-gua, that are directly

868   involved in mutagenicity and carcinogenicity. Otherwise, the epoxides undergo the pathway

869   forming adducts with proteins and phospholipids via the intermediate metabolite, aflatoxin-

870   dialdehyde, to exert toxicity by interfering with the functionality of these macromolecules (Figure

871   1). Conversely, the epoxides are detoxified, mainly via glutathione-S-transferases (GTS) or AFAR

872   mediated conjugation with glutathione or glucuronic acid, respectively. Based on these

873   mechanisms, chemoprotective strategies have been developed with the use of phase II enzyme-

874   inducers to shift the equilibrium of the reactions towards detoxification endpoints (e.g., aflatoxin-

875   GSH, aflatoxin-mercapturic acid, and aflatoxin-glucuronide) that will ultimately be excreted in

876   urine, bile, and/or feces. Various natural and synthetic compounds, including phenolic antioxidants,

877   indoles, isothiocyanates, coumarins, flavones, allyl sulfides, dithiocarbamates, and dithiolethiones

878   were shown to modulate the metabolism of carcinogens by this mechanism [143]. Many of such

879   compounds exert a chemoprotective effect against AFB1-induced liver tumors through the

880   induction of the genetic expression and catalytic activities of phase II enzymes [127,167-168]

881   (Figure 3). Oltipraz [4-methyl-5-(2-pyrazinyl)-1,2-dithiole-3-thione], a synthetic derivative of the

882   natural dithiolethione, is one of these compounds that has received the greatest interest. Its

883   chemoprotective effect has been extensively demonstrated in animals and was shown to be mainly

884   related to the induction of GTS activity as evidenced by the decrease in aflatoxin-albumin and

885   aflatoxin-gua adducts in the serum and urine, respectively [115,169-173]. This chemoprotective

886   agent also inhibits phase I cytochrome P450 enzymes, mainly CYP1A2 and CYP3A4 [143,174],

887   resulting in a decrease of aflatoxin-epoxides and AFM1 levels (Figure 3). The positive outcome

888   of animal studies has encouraged clinical trials with oltipraz in the Qidong region of China where

889   exposure to aflatoxins and HCC incidence are among the highest in the world. Oral administration

890   of oltipraz to people at high risk in this region demonstrated its ability to drive the metabolism of

891   AFB1 towards the detoxification pathway, providing a proof of principle for the aflatoxin-

892   detoxifying effect of this drug in humans [144]. The administration of oltipraz at a daily rate of

893   125 mg induced a 6-fold increase in the excretion of aflatoxin-mercapturic acid in the urine

894   compared with placebo controls, indicating a significant induction of GST. No such a trend in

895   aflatoxin-mercapturic acid excretion was observed when the drug was administered at 500 mg

896   weekly, suggesting that high doses of oltipraz inhibit phase I enzyme, thereby restricting epoxide

897   formation and hence the subsequent steps of the pathway (Figure 3).

898

Aflatoxin B1 ----------➤ Aflatoxin M1

CYP1A2

CYP1A2, CYP3A4

Aflatoxin-epoxides

Oltipraz

GST

Aflatoxin-DNA

Coumarin

Aflatoxin-dialldehyde

Aflatoxin-GSH

AFAR

Aflatoxin-albumin

Aflatoxin mercaturic acd

Aflatoxin-dialcohol ———➤ Aflatoxin-glucuronide

899

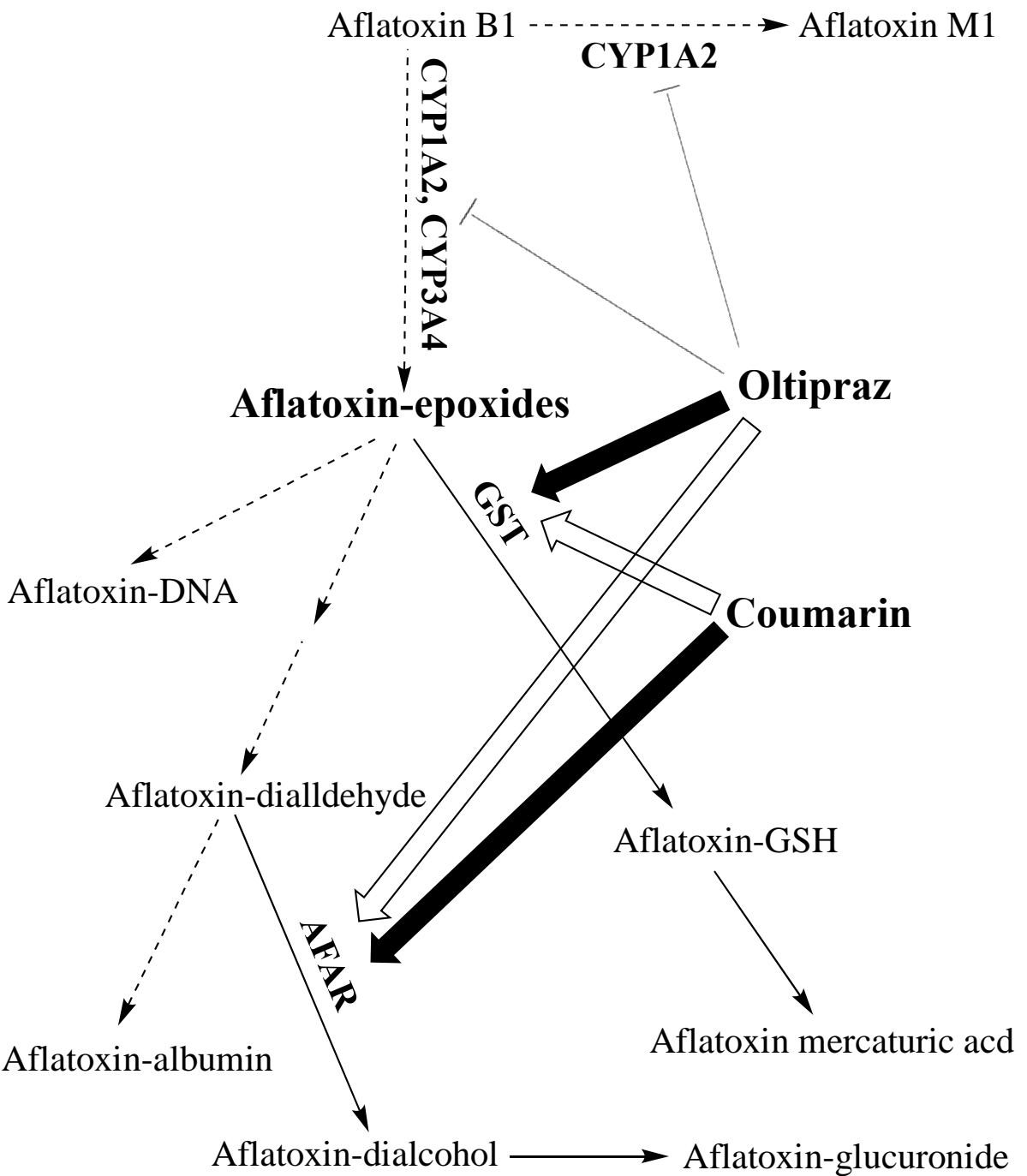



Figure 3: Action of oltipraz and coumarin on the metabolism of aflatoxin B1 against aflatoxin-induced hepatocarcinogenicity. The dominant action of each agent is indicated by a large filled arrow while a lesser inducing effect is indicated by large hallow arrows; T-shaped lines indicate inhibition of the enzymes; dashed arrows indicate reduced rate of the reaction; plain arrows indicate the routes favored by the action of the inducers [127,143]. (For abbreviations, see captions of Figure 1)

906   Despite the proven efficacy of drug-based chemoprotection strategies using natural or

907   synthetic inducers of phase II-enzymes in mitigating the toxicity of aflatoxin, its practical

908   implementation to large-number populations face significant challenges. The use of drugs as part

909   of the regular diet represent a burden to the household budget, especially in target resource-poor

910   settings in developing countries. Additionally, such a practice is perceived by consumers, who lack

911   awareness of the high risk they incur, as a culturally unacceptable deviation from their culinary

912   habits. To address these challenges, government actions aiming to ensure compliance and

913   deliberate involvement of populations are required through the allocation of budgets for incentives

914   and subsidies, organization of training sessions, education, and effective communication

915   campaigns to increase awareness [175]. This is essentially a political decision that may not be

916   feasible or sustainable in many developing countries for insufficiency of budgets and/or the

917   ranking among national priority action plans.

918   Gradual introduction in the diet of specific foods or food-extracts rich in natural inducers

919   of aflatoxin-detoxifying enzymes may be feasible and culturally more acceptable in most countries,

920   especially with the new trend of consumer preferences for drug-free, diversified, and natural foods.

921   Foods rich in bioactive phytochemicals (e.g., glucosinolates, sulforaphanes, polyphenols, and

922   ascorbic acid) are promising sources to substitute drug-based chemoprevention strategies [149].

923   Green tea and members of the cruciferous vegetable family are good candidates owing to their

924   high contents in these compounds [176-177]. The detoxifying action of phytochemicals on AFB1

925   through the induction of phase-II detoxifying enzymes and the inhibition of the aflatoxin-

926   activating phase-I enzymes have been demonstrated in animals as well as by clinical trials [178-

927   180].

928   Green tea polyphenols (GTP) were demonstrated to have an anti-aflatoxin mutagenic effect

929   on *Salmonella typhimurium* [176] and to inhibit the initiation of aflatoxin-induced

930   hepatocarcinogenesis in rats [181]. Surveys have reported an inverse relationship between the

931   level of green-tea consumption and the risk of cancer development in humans [182-183]. A clinical

932   trial involving highly exposed residents to aflatoxins from Guangxi Zhuang Autonomous region

933   of China who were given commercial GTP at two doses (500 or 1000 mg) or placebo four time

934   daily for three months showed that the treatment resulted in a significant increase of aflatoxin-

935   mercapturic acid concentration in urine after the first month, suggesting a decrease in aflatoxin-

936   DNA adduct formation [184]. Further clinical studies are, nonetheless, needed to be conducted on

937   a larger scale to confirm the extent of chemoprotective action in humans, safety, and the form and

938   dose of delivery with regard to the well-known instability of polyphenols and their anti-nutritive

939   properties due to interactions with proteins and micronutrients [185-186].

940   In broccoli, sulforaphane (4-methylsulfinylbutyl isothiocyanate), a well-recognized potent

941   anticarcinogen, was also demonstrated to deviate the metabolism of aflatoxins towards the

942   detoxification pathways by upregulating GTS enzymes [179]. Broccoli is particularly rich in

943   glucoraphanin, the precursor of sulforaphane (SFN) that it releases upon the action of myrosinases

944   [187-188]. The high potency of SFN in preventing cancer diseases [178-180] makes it a favorite

945   candidate for plant-based chemoprotection strategies against aflatoxin-induced

946   hepatocarcinogenicity. Among cruciferous vegetables, broccoli is by far the main source of this

947   phytochemical compound whose precursor glucoraphanin can be extracted by simple water

948   infusion owing to its hydro-solubility [189]. This precursor (glucoraphanin) can be transformed

949   into the active SFN by the action of myrosinases, also present in the plant. Three main ways have

950   been suggested for the application of SFN in chemoprotection strategies:

951   -   A broccoli-rich diet to increase the dietary intake of glucoraphanin which is converted

952   into active SFN in the gastrointestinal (GI) tract by the action of the accompanying

953   myrosinases or by gut microbiota. However, for a maximum benefit, broccoli should

954   be eaten raw. Cooking destroys the heat labile myrosinases and the activation of

955   glucoraphanin will then depend solely on the gut microbiota. This raises bioavailability

956   issue due to the inconsistent activation of the glucoraphanin depending on individuals'

957   microbiota that varies greatly with the age, health status, medications possibly taken in

958   conjunction with the intervention, diet, etc. [190]. In addition, the content of

959   glucoraphanin in broccoli also varies highly with the cultivar and variety, the stage of

960   maturity, the part of the plant eaten (stem or sprout), and storage conditions [177]. This

961   leads to inconsistent and unpredictable results of SFN-based chemoprotection strategy.

962   -   Use of water infusions from young sprouts (3-days old) as the easiest and cheapest

963   extraction way while offering a practical means of delivery as a drink for a given

964   number of times during the day. Here again, the instability of myrosinases to heat

965   during infusion in hot water is a limiting factor leading to complete reliance on the gut

966   microbiota for the activation and bioavailability, and hence to inconsistent outcome.

967   -   Use of food supplement preparations containing pure lyophilized SFN obtained by

968   enzymatic treatment of broccoli infusions with myrosinases [189-190]. Due to the high

969   reactivity and instability of SFN, stabilization treatments are required to preserve its

970   biological activity and bioavailability upon storage under normal environmental

971   conditions (ambient temperature and humidity). A recent study showed that SFN was

972   stabilized by complexation with the food-grade α-cyclodextrin, and it preserved its

973   total biological activity for extended periods at relatively high storage temperatures (22

974   and 37°C) [189]. Commercial food supplements enriched with pure SFN claiming to

975   have anti-cancer properties are already in the market, but their efficacies and means of

976   delivery (oral, topical, or a combination of both) for optimal action are as yet to be

977   soundly validated by clinical studies [191]. Ongoing clinical trials on SFN and those

978   that have already been conducted with outcomes can be visited at

979   https://www.clinicaltrials.gov (accessed July 28, 2019).

980   Other phytochemicals may have potential applications in plant-based chemoprotection

981   strategies against aflatoxin-induced liver cancer, but they have been poorly investigated or are still

982   to be discovered given the increased scientific interest in curative or prophylactic phytotherapy.

983   For example, coumarin, a natural benzopyrone, was shown to be a potent inducer of AFAR, GTS,

984   and NAD(P)H quinone oxidoreductase, known for their anti-oxidative stress action [127]. The

985   study showed that AFAR activity was increased by up to 400-fold in male rats fed on coumarin-

986   containing rations (0.5%) surpassing the effect of two other natural chemoprotective agents, benzyl

987   isothiocyanate and indole-3-carbinol, which caused 5- to 7-fold increase in the activity of the

988   enzyme. Meanwhile, the same coumarin-added diet had a lesser effect, although significant, on the

989   activity GTS that they increased by 10 to 65 times. These results suggest that coumarins may have

990   an additive effect with GTS-inducer phytochemicals against the tumorigenic activity of aflatoxins

991   and are hence potential candidates for multi-agent use in chemoprotection strategies. However, the

992   interactions between different chemoprotective agents, their toxicities and side effects, and means

993   and doses of delivery in humans and animals should to be given full attention before considering

994   their practical application.

995   Another plant-based chemoprotective strategy against aflatoxin-induced cancer using

996   chlorophyll (chl) and derivatives, such as the natural metabolites chlorophyllides (chlide) and

997   pheophorbides (pho) [192-194], and the semi-synthetic chlorophyllin (chln) is attracting increased

998   commercial and scientific interest [192,195]. The antimutagenic and antigenotoxic activities of chl

999   have long been known, and its anti-carcinogenicity has been extensively demonstrated in

1000   experimental animals or *in vitro* on cancer cell-lines [193,195-200]. Chlorophyll is a ubiquitous

1001   constituent of green leafy plants and algae that form substantial components of the human diet,

1002   and a diet rich in green vegetables was suggested to provide the necessary amount of chl for an

1003   effective chemoprotection [195,199]. However, as a food supplement, the effect of chl is

1004   drastically restricted by its high instability and water-insolubility [201]. To circumvent these

1005   limitations, semi-synthetic preparations called chlorophyllins (chln) have been produced and are

1006   commercially available as food supplements under different trade names. Chln preparations are

1007   also permitted as food coloring additives under the E number of E141 (http://www.food-

1008   info.net/uk/e/e141.htm, accessed July 31, 2019). These are sodium-copper salt derivatives of

1009   chlorophyll consisting mainly of mixtures of disodium copper chlorin e4 and trisodium copper

1010   chlorin e6, which derive from chlorophyll A by substituting the magnesium ion of the porphyrin

1011   ring (chlorin) for a copper ion and removing the hydrophobic phytol tail (Figure 4). This provides

1012   chlorophyllin with the desired stability and water solubility while keeping the functional properties

1013   of the parent chlorophyll. *In vivo* studies on chemoprotective effect of chlorophyll and

1014   chlorophyllin showed that they were both potent anticarcinogens with high potential for practical

1015   application to alleviate the liver-cancer incidence [197-198]. Chlorophyllin was shown to induce

1016   *in vitro* cell cycle arrest and apoptosis [200], and to exert a cytotoxic effect on various cancer cell

1017   lines, although to a lesser extent than chl and chlide [193]. The administration of 100 mg of chln

1018   three times a day for 4 months to Qidong residents highly exposed to unavoidable dietary

1019   aflatoxins, has resulted in a-55% decrease in the median urinary concentrations of aflatoxin-gua

1020   adduct compared with those recorded in placebo controls [202]. As regards the mode of action of

1021   chlorophyll and derivatives, most studies tend to concur that they act by directly complexing and

1022   trapping the carcinogen regardless of its species thereby reducing its bioavailability and systemic

1023   absorption [192,197-199]. Other studies, however, showed that they may act in different manners

1024   depending on the chl derivative. For example, while chlide reduced the formation of aflatoxin B1-

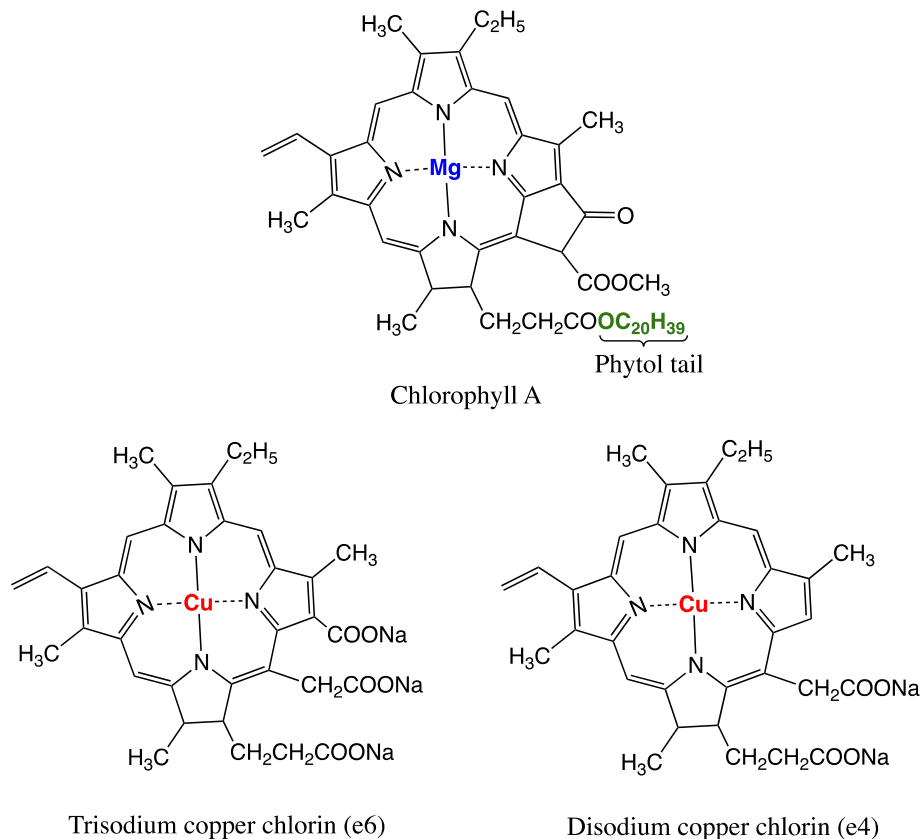
1025   gua adduct by trapping the toxin via the complex aflatoxin-chlide formation in murine hepatoma

1026   cells, pho additionally induced phase II GTS enzymes [192]. At the molecular level, studies on

1027   cancer cell-lines suggest that chl and derivatives act by inactivating signal transduction pathways

1028   [193,200].

Chlorophyll A

Trisodium copper chlorin (e6)   Disodium copper chlorin (e4)

1029
1030   **Figure 4**: Chlorophyll A molecule naturally found in green plants, algae, and
1031   photosynthetic bacteria, and the two main semi-synthetic derivatives, disodium copper chlorin e4
1032   and trisodium copper chlorin e6, commonly used in commercial chlorophyllin food supplements.

1033   *3.3.2.2*   Reducing the risk by interfering with the bioavailability of aflatoxins

1034   Interference with the bioavailability of aflatoxins in the GI tract, by non-digestible food-

1035   grade sorbents or microorganisms has also been considered for chemoprotective strategy

1036   development. Enterosorption of aflatoxins by montmorillonite clay (NovaSil^{TM}; NS), an anti-

1037   caking agent in animal feeds, was shown to remove efficiently aflatoxins and reduce their toxicity

1038   in rats without noticeable side effects [203]. The safety of uniform particle size of NS (UPNS) and

1039   its effectiveness in removing aflatoxins in humans were also demonstrated by clinical trials on

1040   volunteers from populations highly exposed to aflatoxins through their normal diet [204]. The

1041   levels of AFM1 biomarker in the urine of the volunteers treated with UPNS were reduced by 55%

1042   compared with placebo-treated controls. Unlike drug-based chemoprotection interventions, the

1043   inclusion of clay in the diet would be culturally acceptable in Africa and Asia where clay

1044   consumption was reported to be common in traditional ethnic groups of these regions [205]. In

1045   addition, studies demonstrated the absence of short-term adverse health effects in rats fed on

1046   rations containing up to 2% (w/w) of clay [206] as well as in poultry [207]. The high affinity of

1047   SN clays (montmorillonite and smectite) to aflatoxins suggests that they are unlikely to interfere

1048   with the absorption of micronutrients (e.g., metals and vitamins), as their action would be

1049   selectively directed towards aflatoxins [205]. Yet, further studies on long-term side-effects are

1050   needed to substantiate the safety, practicability, consumer acceptability, and risk-benefit profile of

1051   food-grade clays incorporation into the diet.

1052   The enterosorption of aflatoxins in the GI tract to reduce their bioavailability can also be

1053   achieved by resident microbiota of the intestinal tract. Supply of probiotic bacteria with aflatoxin-

1054   binding properties to high-risk consumers in the form of encapsulated preparations or as biological

1055   supplements in fermented foods has also been recommended [208]. Lactic acid bacteria commonly

1056   used in meat, vegetable, and dairy fermentations have been reported to bind and remove aflatoxins

1057   [209-211]. The "generally recognized as safe" status of these microorganisms and their worldwide

1058   use in food fermentations for millennia is an evident advantage with respect to their practicability

1059   and consumer-acceptability. Lactobacilli strains have received the most attention for their

1060   probiotic properties, including aflatoxin-bioavailability reduction, and for their reputed ability to

1061   colonize the intestinal tract. A particular focus was made on *Lb. rhamnosus* GC strain, which was

1062   demonstrated not only to bind and excrete aflatoxins in the feces causing the removal of up to 80%,

1063   but also to alleviate the associated liver injury and growth faltering in rats [210,212]. Other

1064   lactobacilli strains of different species grown under controlled conditions in the presence of AFB1

1065   were demonstrated to bind and remove between 25 and 61% aflatoxin within 72 hours; *Lb.*

1066   *fermentum* and *Lb. plantarum* strains could remove instantaneously 56 and 61%, respectively

1067   [213]. Binding and removing AFB1 from a liquid solution at rates varying between 19 to 45%

1068   were recorded with *Enterococcus faecium* strains [214]. However, the results of these *in vitro* or

1069   animal studies are not directly transposable to humans with different metabolism, intestinal

1070   ecological conditions, and immunological response that may not be suitable for the candidate

1071   strains to grow and produce the anticipated effects within the GI tract. To simulate the human

1072   gastrointestinal conditions, an *in vitro* digestion model was designed to test lactobacilli and

1073   bifidobacterial strains for their ability to remove AFB1 from different food matrices, and the results

1074   showed moderate performances with a maximum reduction of 37% [215]. Given the ethical

1075   considerations that preclude administration of aflatoxins to humans in an experimental design,

1076   clinical trials on populations with high dietary exposure to aflatoxins e.g., African and Southeast

1077   China, remains the most appropriate alternative, but they are not always accessible and easy to

1078   perform. In addition, the results obtained on a given population may not apply to other populations

1079   in different parts of the world for genetic, ethnic, and culinary habit reasons. Nonetheless, studies

1080   on these approaches yielded inconsistent results regarding the percentage of aflatoxin

1081   removal, the time necessary for maximum binding, and parentage released back. In

1082   addition, the long-term effect on the equilibrium of the intestinal microbiota with possible

1083   adverse effects remain to be addressed [216].

1084   Apart from the ability of resident intestinal microbiota to bind and remove aflatoxins, they

1085   can also interfere with their bioavailability by enzymatic degradation. Bacteria, such as

1086   *Escherichia coli, Bacillus* spp. *Pseudomonas* spp. *Stenotrophomonas* spp., *Arthrobacter* spp., and

1087   members of the family *Flavobacteriaceae* were shown *in vitro* to produce aflatoxin-degrading

1088   enzymes causing a significant reduction (>90%) in the levels of AFB1, AFM1 and/or AFB2 [217-

1089   220]. However, for these microorganisms to be effective *in vivo*, they have to colonize the

1090   intestinal tract and express sufficient levels of the aflatoxin-degrading enzymes. This may require

1091   specific physicochemical conditions and the presence of inducing factors for the enzyme to be

1092   optimally produced and active. A highly active aflatoxin-degrading enzyme produced by *Bacillus*

1093   *shackletonii* was indeed shown to require specific inducers for their expression and to be degraded

1094   by proteinase K which, in addition to the aerobic character of the producing bacterium, limit

1095   drastically its potential use as a probiotic strain in a chemoprotective strategy [218]. A suitable

1096   microorganism for a chemoprotection strategy should be able to adapt to the prevailing ecological

1097   conditions of the GI tract (pH, temperature, red/ox potential, and oxygen), to withstand the

1098   presence of inhibitory substances, e.g., bile and bile salts, and to compete with co-existing

1099   microorganisms, among other general requirements for a microbial strain to have the probiotic

1100   status [221]. These conditions narrow the scope of finding the ideal microbial strain where all the

1101   requirements are met, and no single strain, to our knowledge, was clinically studied for their

1102   effectiveness in degrading aflatoxins in humans. A suggested alternative was the use of multiple

1103   strains in one probiotic preparation [114,222], but maintaining the viability of the constitutive

1104   strains at appropriate ratios is the most challenging issue. A similar issue is experienced by cheese-

1105   making industry using complex starter cultures; the issue is certainly more problematic in the GI

1106   tract. Clinical trials were conducted on individuals from the population of Guangzhou, another

1107   southern Chinese region known for its high dietary intake of aflatoxins, to assess the efficacy of

1108   chemoprotection strategy using a combination (1:1) of two aflatoxin-binding probiotic bacteria

1109   (*Lb. rhamnosus* LC705 and *Propionibacterium freudenreichii* subsp. *shermanii*) [222].

1110   The performance was assessed by monitoring aflatoxin-gua concentration in urine samples

1111   collected regularly during 5 weeks of treatment and 5 weeks after the treatment was ceased

1112   (a total of 10 weeks for the trail). A gradual decrease was observed in the mean urinary

1113   aflatoxin-gua concentration of the intervention group (receiving the probiotic capsules

1114   twice a day) compared with placebo group (receiving cellulose capsules) to reach a

1115   maximum of 55% reduction. Interestingly, no significant difference was observed in

1116   urinary concentration of the aflatoxin-gua biomarker between the two groups 5 weeks after

1117   the treatment termination, suggesting that the bacterial strains could not colonize the gut to

1118   become a part of its normal microbiota and sustain the modulatory effect on the toxicity of

1119   aflatoxins.

1120   A wide range of chemoprotection strategies based on the use of natural or synthetic drugs,

1121   foods and food constituents, and binders (microorganisms or inorganic non-nutritive materials)

1122   have been studied for their ability to provide some degree of protection against liver cancer caused

1123   by aflatoxins. However, none of them has been conclusively established as a universal application

1124   or shown to actually provide effective long-term chemoprotection for large-number populations.

1125   Plant-based chemoprotection appears to be promising, acceptable by consumers, and cost-effective,

1126   but there is still much to do in scientific research to soundly demonstrate its effectiveness, safety,

1127   and practicability. Combinations of different strategies or chemoprotective agents with different

1128   and complementary modes of action remain to be examined. Research in this field is seriously

1129   hampered by difficulties in performing clinical trials and the ethical prohibition of conducting

1130   experiments on human volunteers to whom aflatoxins should be administered. Most of the relevant

1131   clinical trials are conducted in only two Chinese regions, Qidong and Guangxi, where the dietary

1132   exposure to aflatoxins is unavoidably high, which may not be representative of the rest of the world.

1133   The development of reliable biomarkers for the prediction of aflatoxin-induced cancer early

1134   enough to allow testing safe and ethically-acceptable strategies before the disease onset may help

1135   advance the scientific and applied research on chemoprevention. Moreover, the modern lifestyle

1136   with a clear trend of increased consumption of alcohol, tobacco, and fast and junk foods adds new

1137   challenges and risk factors that should be taken into consideration in scientific research on

1138   aflatoxins for future decades [1,149,223].

1139   *3.4   Legislative challenges*

1140   The advent of globalization with the establishment of the WTO and the SPS agreement

1141   created the need to harmonize regulatory provisions for the flow of the global trade. Aflatoxins

1142   regulations have been one of the most challenging issues facing harmonization, as they are highly

1143   divergent throughout the world. While some countries set stringent standards to ensure the highest

1144   protection possible to public health, others have adopted more permissive standards aiming at an

1145   acceptable balance between "food safety" and "food availability" or to allow simplified trading

1146   with economic partners [224]. On the other hand, many countries have not promulgated yet

1147   aflatoxin regulations due to the associated high costs (food loss/condemnation, inspection,

1148   sampling, analyses, control at the borders, etc.) [159] and to insufficient institutional and capacity

1149   building for rigorous enforcement [224]. As a result, aflatoxins are inconsistently regulated across

1150   the globe regarding the aflatoxin types, food and feed commodities, and the standards in each item

1151   or group of items [224-225]. Depending on the country or region, the maximum tolerable limits

1152   (MTL) or the maximum limits (ML) can be set for AFB1 and/or for total aflatoxins (sum of AFB1,

1153   AFB1, AFG1, and AFG2); some countries set MLs for AFM1 in milk, dairy products, and/or infant

1154   formula. The MLs of the regulated aflatoxins are set for specific foods or foods in general, or for

1155   specific   feeds   or   feeds   in   general,   depending   on   the   country   or   region

1156   (http://www.mycotoxins.info/en/regulations/, accessed on July 7, 2019). The general tendency of

1157   the standards is to be strict in industrialized countries and relaxed in developing countries,

1158   especially those of the tropics facing the highest problems to control aflatoxin contaminations.

1159   This lack of uniformity, added to the conflicting economic interest of each country, complicates

1160   greatly the harmonization process, especially when stringent regulations are imposed to

1161   developing countries or when scientific data to conduct meaningful risk assessment are missing or

1162   controversial. Nonetheless, harmonization has been fairly easy to reach in countries of the same

1163   geographical area and with comparable levels of development, such as the European Union (EU)

1164   countries, Australia and New Zealand, North America (USA and Canada), and South American

1165   countries members of the Southern common market (MERCOSUR), i.e., "Mercado Cómun del

1166   Sur". At the global level, standards of each aflatoxin-commodity are matter of debate under the

1167   auspices of the *codex alimentarius commission* (CAC) before acceptance in the CA. The debate

1168   generally opposes industrialized, especially the EU, to developing countries. The first side claims

1169   strict standards to guarantee appropriate and due protection to consumers, and the second

1170   advocates relaxed standards to ensure fair trade and offer more economic opportunities to

1171   developing countries while maintaining an adequate level of safety [226]. It is generally admitted

1172   that imposing strict standards to developing countries affects severely health and economy, as they

1173   tend to export products of the highest quality and keep those of poor quality (high aflatoxin-

1174   contamination) for local consumption, which increases the exposure and hence health risk to local

1175   communities [226]. It has been argued, however, that strict standards with proper enforcement

1176   measures and international cooperation may benefit developing countries by forcing them to

1177   improve gradually the quality of their products and gain expertise through their interactions with

1178   industrialized countries, i.e., a "forcing technology" approach [227-228]. However, from the SPS

1179   agreement standpoint, unless strict standards are scientifically justified by health risk

1180   considerations, they would be regarded as prohibited technical barrier to trade. A study

1181   demonstrated that relaxing the ML of the total aflatoxins in tree nuts in the EU from 4 µg/kg to 10

1182   µg/kg in 2009 benefited both EU-members and exporters from developing countries without undue

1183   increase in public health risks [226]. The two authoritative bodies for risk assessment studies, the

1184   FAO/WHO joint expert committee on food additives (JECFA) and the European food safety

1185   authority (EFSA), agreed that this shift would not adversely affect the public health. Conversely,

1186   a similar request to relax the UE standards of total aflatoxins in ready-to-eat (RTE) peanut from

1187   the current ML of 4 µg/kg to 10 µg/kg was refuted on the basis of EFSA risk assessment study

1188   suggesting that this change would cause an unacceptable additional increase of the risk by a factor

1189   of 1.6 to 1.8 [229]. The latter conclusion contrasts with the outcome of the JECFA assessment

1190   suggesting that an increase of ML from 4 µg/kg to 10 µg/kg will cause a marginal or no increase

1191   in the risk, but would reduce the rejection rate by more than 7% [230]. This reduction in the

1192   rejection rate was estimated to save about 233,333 metric tons of RTE peanut, corresponding to a

1193   trade value of about US$ 327 million [231]. The UE criticized the outcome of the latter study

1194   mainly from the standpoint of the sampling procedure and the regions of the world considered to

1195   determine the exposure. Similarly, a study conducted in Kenya demonstrated that the strict

1196   enforcement of the national standards for AFB1 in grains (maize, millet, and sorghum), and AFM1

1197   in milk would deprive 9 million Kenyan consumer from their staples, and about 3.4 million

1198   consumer from milk; strict application of AFB1 standards in feeds was also suggested to result in

1199   a loss of 336,217 kg of milk [224]. Such a situation has been experienced in Serbia where setting

1200   the ML of AFM1 in milk to 0.05 µg/kg, for harmonization with the EU regulations, caused a major

1201   recall (62.3%) of the milk produced in 2013, urging the country to relax back the standards to 0.5

1202   µg/kg next year [232].

1203   Harmonization of aflatoxin regulations is mandatory to avoid litigious situations between

1204   member states of the WTO, but it will continue to raise challenges for at least the two next decades

1205   due to the highly divergent views on political economy, risk perception, and for scientific reasons.

1206   In addition to the lengthy procedure of the negotiations on a case-by-case basis within the codex

1207   committee on contaminants in foods (CCCF) and the codex alimentarius commission (CAC).

1208   Countries with high-income and larger population tend to rise food safety issues and make

1209   lobbying efforts to impose standards that benefit their competitive economic sectors and provide

1210   them with tariffs and trade advantages; for a thorough discussion of this matter, see [233]. Use of

1211   science to shape regulatory provisions without bias is not always possible because of shortages in

1212   scientific criteria or data gaps to perform a sound quantitative risk assessment with undisputable

1213   conclusions. The current case of LM for aflatoxins in RTE peanut, discussed above, is one of the

1214   many cases that are being considered by the CCCF with difficulties to reach a consensus. Apart

1215   from the lack of reliable data of exposure in many parts of the world and for different groups of a

1216   population, sampling plans and analytical techniques to reach a risk assessment of a commodity-

1217   hazard combination have been criticized [227]. In addition, the characterization of the risk related

1218   to aflatoxins has not been defined with certainty; the exact genotoxic dose of aflatoxins is not

1219   known and depends on various factors including the type of aflatoxin, individuals or group of

1220   individuals, and co-occurrence with other mycotoxins. It is being increasingly evident that co-

1221   occurrence of mycotoxins in foods and feeds is a very common phenomenon, and they interact

1222   with each other to produce toxicological effects that differ markedly in intensity and outcome from

1223   those produced individually [234]. This predicts the rise of new challenges related to the regulatory

1224   status of known or as yet to be discovered mycotoxins that may co-occur with aflatoxins possibly

1225   interfering with their toxicities, and which may require to be regulated in their own right.

1226   Intensive scientific research is being conducted to address these challenges and provide

1227   new scientific information on aflatoxins to regulatory authorities. However, seeking the highest

1228   protection possible against aflatoxin intoxications by strengthening the standards may result in

1229   only a marginal reduction of the associated health risks while inducing excessive economic losses

1230   and food shortage with serious nutritional health consequences [226,234]. Although this is an

1231   international development issue which requires actions at different socio-economic and political

1232   levels, scientific research is a key instrument to define the borderline between objective science-

1233   based food safety regulatory provisions, on one hand, and socio-economic and international trade

1234   considerations, on the other hand.

1235   **4   Conclusions**

1236   Since their discovery, aflatoxins have gained increased scientific interest due to their high

1237   impact on health, economy, and social life. During the sixties, scientific research on aflatoxins,

1238   which has been focused on their chemical characterization and toxicity-testing generated

1239   appreciable information of different types of aflatoxins and their association with liver cancer in

1240   various animals. This has initiated a large debate internationally about their hepatocarcinogenicity

1241   in humans and emphasized the need for their regulations in different commodities to secure food

1242   safety and ensure the flow of the trade. This debate has further stimulated research on aflatoxins

1243   aiming at improving the experimental design of clinical and epidemiological studies in order to

1244   yield sufficient and convincing evidence for aflatoxin carcinogenicity in humans. Biosynthesis and

1245   biodegradation pathways, and the mechanism of action were also intensively investigated to

1246   support the causal link between aflatoxins and liver cancer. Periodically, the IARC working group

1247   has performed critical reviews of the newly obtained results and appraised the advances made in

1248   the knowledge of these mycotoxins with regard to their health impact ultimately leading to the

1249   definite establishment of their carcinogenicity via genotoxic action. It was also established that the

1250   genotoxicity of aflatoxins is enhanced by chronic HB virus infection, although the mechanism of

1251   this synergy remains to be demonstrated.

1252   In the meantime, sustained efforts have been deployed worldwide to limit the health risks

1253   associated with aflatoxins by reducing the exposure via dietary intake. The use of high-tech

1254   agricultural management system allowed industrialized countries to tackle efficiently the problem

1255   and provide a reasonable protection to their consumers, which has encouraged them to promulgate

1256   increasingly stringent standards. In contrast, most developing countries, especially the tropical and

1257   sub-tropical continue to struggle with the high incidence of aflatoxin contamination of foods and

1258   feeds due to their economic vulnerability and the favorable climatic conditions for aflatoxigenic

1259   mould growth and aflatoxin production. Therefore, most of these counties are unable to adopt and

1260   enforce strict standards, which limits their access to the markets of industrialized countries and

1261   creates an imbalance in agri-food international trade. This issue also continues to be a matter of

1262   debate within the WTO to avoid disguised use of food safety regulations as a technical barrier to

1263   fair trade. The SPS agreement emphasizes the need for science-based regulatory provisions

1264   standards using risk assessment as the scientific basis to determine the standards to protect human

1265   and animal health while facilitating global trade of agri-foods. However, food safety risk

1266   assessment is a relatively recent discipline and does not provide indisputable answers to all issues

1267   raised due to the lack of the necessary data, e.g., exposure and/or the exact toxic dose for many

1268   hazards, including aflatoxins. This debate will continue to challenge the international community

1269   for decades to come awaiting the generation of meaningful risk assessments of aflatoxins as

1270   standalone hazards and/or as part of multi-mycotoxin contaminant in foods and feeds.

1271   Despite the scientific progress in the knowledge on aflatoxins and the efforts made to

1272   reduce the risk they pose to public health, developing countries still have to tolerate high level of

1273   contamination of foods and feeds to not compromise food supply. Therefore, alternative ways to

1274   mitigate health risk by detoxifying already contaminated foods and feeds before consumption or

1275   by acting on consumers to interfere with aflatoxin toxicity or bioavailability *in situ* have been

1276   suggested. All proposed interventions have yielded inconsistent results to provide definite and

1277   practicable solutions at large scale, and further refinements are needed before their efficacies and

1278   conditions of their use are recognized. The impact of the presently suggested intervention

1279   approaches will be complicated in the future by the climatic change with a clear trend to enhance

1280   the risk of aflatoxins as well as the change in the life style that introduces additional risk factors.

1281   In fact, it is evident that reducing aflatoxin contamination and associated health risks cannot be

1282   achieved by specific interventions, but rather by concerted actions at many levels and by all

1283   stakeholders. These actions should include education, scientific research, legislation framework,

1284   environment protection to limit climatic change, and international cooperation for sustainable

1285   development to fight hunger and poverty. Partnership and research programs on aflatoxins should

1286   be reshaped to be oriented towards practicable solutions with greater involvement of social

1287   scientists. The formation of research and association networks between developing and

1288   industrialized countries would play a paramount role in creating a synergy to ensure exchange

1289   information and technology transfer and bridge the gap between North and South of the globe.

1290

1291   **Acknowledgment**

1292   The author declares has no conflict of interest

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