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Review

Application of the assay of aflatoxins by liquid chromatography with fluorescence detection in food analysis

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Abstract

HPLC using fluorescence detection has already become the most accepted method for the determination of aflatoxins due to its several advantages over other analytical methods. Both normal- and reversed-phase HPLC can be used. However the reversed-phase HPLC methods are more popular. Liquid chromatographic determination of aflatoxins using fluorescence detection and its application in food analysis is reviewed in this article.

Keywords: Food analysis; Aflatoxins

Contents

1. Introduction

In the 1960s, 100 000 turkeys were lost in the UK as a consequence of an intoxication due to toxic metabolites coming from fungi [1,2]. The cause of this disease was traced to a feed component, peanut meal which was infested by *Aspergillus flavus*, a fungi that commonly grows in this substrate during its storage. The analysis of this feed revealed a series of fluorescent compounds that were recognized as mycotoxins and denominated aflatoxins (AFs). At the same time, in the USA, an outbreak of hepatoma was

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observed in the rainbow trout which later on was related to the contamination with aflatoxins of a mixture of cotton seeds that was a component of the diet of these fish [3].

The ubiquity of the aflatoxin-producing fungi and the potent biological activity of their mycotoxins at very low concentrations has stimulated a phenomenal amount of research in many different fields [4–15]

Many aflatoxins exhibit acute and chronic toxicity including mutagenic, carcinogenic and teratogenic effects in a wide range of organisms [16–20]. The International Agency for Research on Cancer has placed AFB_1 on their list of probable human carcinogens [21].

Although susceptibility of humans to aflatoxins in not well known, results of epidemiological studies carried out in Africa and Asia, where there is a high incidence of hepatoma, have revealed an association between cancer incidence and the aflatoxin content of the diet [22–24]. The most severe outbreak of human hepatitis due to aflatoxicosis was registered in India in 1974, where 108 of 397 patients that had consumed contaminated corn with aflatoxins levels from 0.25 to 15 mg/kg died. Also the illness known as 'cirrhosis of Indian childhood ' is partly due to an aflatoxic poisoning. The 'Reye's syndrome', with encephalophatias and fatty degeneration in the viscera of children, has also been related to the toxicity of aflatoxins [25,26]. In Thailand, where it is endemic, aflatoxins B_1, G_1 and B_2 have been detected by chromatography in the human liver [27] and presence of metabolites P_1 and Q_1 in this tissue, has been related to cancer [28].

The widespread occurrence of the *Aspergillus* mould and the high carbohydrate content, means several agricultural commodities are vulnerable to contamination with these type of moulds [29]. The storage of these products under high moisture and increased temperature (25–30°C) favours the natural occurrence of aflatoxins in a great variety of foods for animal and human consumption. Aflatoxins have been found in several human foods like barley [30] corn [29–39], rice [31], beans [30,40,41], brazil nuts [42], pistachio nuts [43–45], peanut and peanut products [30,31,44–50], almonds [30,51], nut and nuts products [49], wheat flour [30,52], figs [53], date fruits [54], spices [55–57], eggs [58], beer [59], etc. Due to the metabolism of these mycotoxins, the consumption of polluted feed is also derived from the contamination of foods of animal origin such as meat, milk and other dairy products. AFM_1 acummulates in the milk of cows that had consumed feed contaminated with AFB_1 [14,60–75] This compound presents the same toxicity of AFB_1 for animals [29] and humans [60,76,77].

Some *A. flavus* varieties produce aflatoxins: *A. flavus* var. *flavus*, *A. flavus* subsp. *parasiticus*, and *A. nomius* share the ability to produce aflatoxins. Up to now, at least 16 aflatoxins [78] that are related structurally have been identified (Fig. 1). Among them, the G series of aflatoxins differ chemically from the B series by the presence of a 3-lactone ring, instead of a cyclopentenone ring. Also, an 8,9 double bond is found in the form of a vinyl ether at the terminal furan ring in AFB_1 and AFG_1, but not AFB_2 and AFG_2 [79,80]. However, this small difference in structure is associated with a very significant change in activity; whereby AFB_1 and AFG_1 are carcinogenic and considerably more toxic than AFB_2 and AFG_2 [81]. Aflatoxins more frequently analysed are: B_1, B_2, G_1,and G_2, due to their well known pathogenic action, and the hydroxylated metabolites of AFB: M_1, M_2 that can be found in milk and other organic fluids [82–84] and Q_1 and P_1 that can be present in the liver or urine of many mammals, including humans [4,28,76,85].

From a chemical point of view, the highly conjugated and rigid aflatoxin moieties give rise to native fluorescence characteristics of those kind of compounds. Moreover, it should be noticed that the small structural variations distinguishing the aflatoxins have a drastic influence on the cited fluorescence properties i.e: G_2 and B_2 derivatives are far more fluorescent than their unsaturated homologues: B_1 and G_1.

Consequently, the aim of the present review is to give an overview of the analytical methods based on this spectroscopic characteristic of aflatoxins, especially after coupling with HPLC, that are applied to aflatoxin determination in food.

2. Chromatographic methods for aflatoxin analysis in foods

2.1. Previous treatments

Chromatographic analysis of aflatoxins is preceded

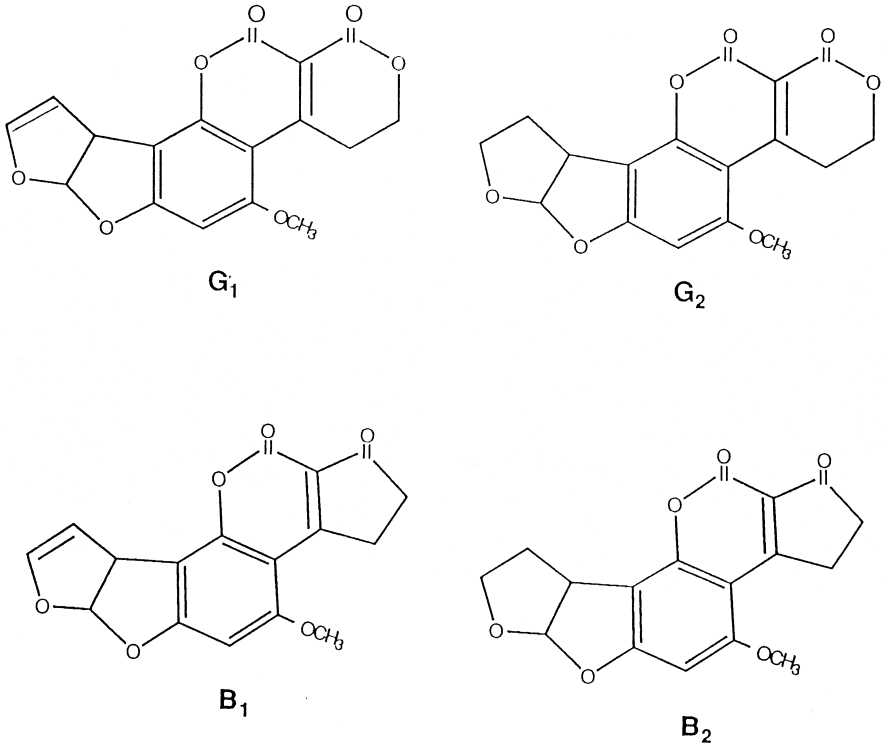

Fig. 1. Chemical structure of the main aflatoxins.

by a sequence of broad and complex general operations that include sampling, sample preparation, extraction, purification and concentration of the extract obtained before the separation, quantitation and confirmation steps. The results of the later quantification and identification steps are determined by the effectiveness obtained in the general operations [85].

2.1.1. Sampling and sample preparation

Sampling — selection of a representative sample from the population for analysis, is the most important aspect of analysis since the aflatoxins are not uniformly distributed in the foodstuff. Sample preparation of products such as cereals, nuts or feeds, involves reduction of the particle size to increase the surface area and decreasing the mean free path of extraction solvent, then the sample can be effectively mixed for homogeneity and subsampling [86,87]. For liquids and pastes, adequate mixing is required before subsampling [88].

2.1.2. Extraction

Extraction depends to a great extent on the physicochemical properties of the commodities contaminated with aflatoxin [88]. Due to the diverse nature of products that can be contaminated, there is not a single adequate method of extraction for all products. Commodities with high lipid and pigment content require a more selective treatment followed by extensive purification methods than those with a low content of these components. As aflatoxins are soluble in slightly polar solvents and insoluble in completely nonpolar solvents, normally they are extracted using mixtures of organic solvents such as acetone, chloroform, or methanol [87]. On the other hand, the use of small amounts of water in combination with the cited solvents humidifies the substrate increasing the penetration of the organic solvent in

the sample and enhancing aflatoxin extraction [88]. The addition of nonpolar solvents, like hexane, for fat partitioning is also used [89].

2.1.3. Purification

When purification and clean-up are required, liquid–liquid partitioning or, more recently, solid-phase extraction cartridges and inmunoaffinity columns are used. Since Eppley in 1968 [90] developed silica-gel purification columns for aflatoxin analysis, a large variety of solid-phase extraction columns have been developed for this purpose. The bonded phase may either be polar (silica cartridges) [91] or non polar (C_{18}, C_8, C_2, cyclohexyl or phenyl cartridges) [83,92]. Other types of solid-phase extraction columns are the multifunctional Mycosep columns that, contrary to the others, allow the passage of mycotoxins and retain the interfering substances [93] and the inmunoaffinity columns. The latter supposes the most modern clean-up tools for aflatoxin analysis [48,53,56,57,71,94–100].

The extracted aflatoxin solution obtained is then collected and concentrated for chromatographic analysis.

2.2. High-performance liquid chromatographic procedures

Although TLC techniques were extensively used for aflatoxin analysis, recently an increase in the use of HPTLC has been noted. The accuracy of TLC is less than that of HPLC [12] but the results obtained using HPTLC are similar to that of high-performance liquid chromatography (HPLC) and more consistent than enzyme-linked immunosorbent (ELISA) data [102]. However, the present trend is the use of HPLC as an election technique for aflatoxin analysis. Its main advantage over other methods seems to be the potential for automation.

Use of HPLC as a determination procedure in the analysis of foods for aflatoxins has greatly increased in recent years and several reviews have been carried out [12,13,88,102–113].

In the first research studies with HPLC [40,114], the normal stationary phase (NP–HPLC) was used coupled with a detection system for UV absorption which was insufficient to determine the aflatoxins at subnanogram levels.

Since aflatoxins have fluorescent properties, it was soon demonstrated that systems for determination with fluorescence detection were more sensitive [115,116]. One of the most important problems is the main aflatoxins (B_1, B_2, G_1 and G_2) fluorescence dependence towards the composition of the solvent. For example, mobile phases used for NP-HPLC, contained chloroform or dichloromethane. Under these conditions, the fluorescence detection at nanogram levels was only possible for AFG_1 and AFG_2 because the fluorescence emission of AFB_1 and AFB_2 was markedly quenched. This made it necessary to simultaneous use an UV detector for AFB_1 and AFB_2 and a fluorescence one for AFG_1 and AFG_2 [117].

Manabe et al. [31] found that the addition of an organic acid to the mobile phase enhanced the fluorescence intensities of AFB_1 and AFB_2. This method, with slight modifications, was used for aflatoxins determination in diverse agricultural products [33], mixed feed [118] and for the determination of AFM_1 and AFM_2 in milk [64].

Zimmerli [119] and Panalaks and Scott [115], on the basis that fluorescence of AFB_1 and AFB_2 was higher in adsorption solids (i.e.: TLC), developed a technique in which the detector flow-cell was packed with microparticles of silica gel where aflatoxins were adsorbed in a reversible way. This technique was applied to a post-column reaction and only a small increment in the peak widths was observed. The sensitivity for AFB_1 and AFB_2 reached, in this way, the levels obtained for AFG_1 and AFG_2. Several authors have applied this method to aflatoxin determination in various agricultural products [30,46,55,120] and for the determination of aflatoxin M_1 and M_2 in dairy products [121]. Fluorescence detection using a packed cell was also applied to increase the sensitivity of AFB_1 and AFG_1 in methanol–water mobile phases for reversed-phase HPLC (RP-HPLC), although the fluorescence signal remained lower than for AFB_2 and AFG_2 [122].

Aflatoxins can be resolved by RP-HPLC columns with methanol–water or acetonitrile–water, but in these aqueous solvents, the fluorescence of AFB_1 and AFG_1 is rather weak [109,123]. In general, RP-HPLC systems are used more frequently than those of normal-phase due to the easier manipulation as well as the smaller toxicity of this watery mobile

phases. Because in these types of solutions, AFB_1 and AFG_1 fluorescence diminishes, different derivatization procedures have been tested including the use of strong acids such trifluoroacetic acid (TFA) and oxidants such as chloramine T [124], iodine and bromine.

Takahashi [116], by means of a pre-column derivatization with TFA, transformed the non-fluorescent AFB_1 and AFG_1 in to their highly fluorescent hemiacetals B_{2a} and G_{2a}. Due to their saturated structure, AFB_2 and AFG_2, are not affected by this conversion. The detection limits reached were 0.02 μg/l for the four aflatoxins. In 1978, Beebe [125] adapted this method to several foods. A liquid–liquid extraction with *n*-hexane associated with derivatization with TFA to liberate the aflatoxins from the extracted waxy residues, was employed. It has been proven that the addition of hexane doesn't interfere with the derivatization procedure. The TFA derivatization method has also been used for determining aflatoxins in a variety of foods [32,34,36,38,43,50,51,82,126]. Since 1980, this method was extended to aflatoxin M_1 determination [127] and used for milk and dairy-products analysis [61,62,65–67,70,97,128–131]. Aflatoxin hemiacetals were also generated with post-column in-line photochemical UV irradiation and this method was applied to corn analysis [37]. Aflatoxin hemiacetals can be detected at subpicogram level using HPLC with laser fluorimetry [132]. The major disadvantage of TFA derivatization is the low stability of B_{2a} and G_{2a} derivatives in methanol probably due to the formation of methyl acetals [125]. It can be partly avoided if this solvent is not used as a component of the mobile phase [109]. On the other hand, Dell et al. [101] found this method precise but biased for the analysis of peanut butter. Perhaps the precolumn derivatization step was the source of this bias.

Furthermore, with automated HPLC systems, post-column derivatization appears preferable in order to reduce the manipulations required on each sample, the relative merits of the TFA methods are counterbalanced since TFA is not the most suitable reagent for post-column derivatization because of its toxicity and its corrosive properties on the pumping devices [133].

Recently, different methods of post column derivatization have been developed. Davis and Diener [134] reported that AFB_1 and AFG_1 give some intensely fluorescent derivatives with iodine. In 1980, these authors developed chromatographic analysis of these AFB_1 derivatives with iodine [135]. In a RP-HPLC system with fluorescence detection, the peak of AFB_1 disappeared, whereas a new peak appeared with an intensity 25 times higher. They used this derivatization only as a checking test since it also leads to the formation of numerous secondary products. Thorpe et al. [136] were the first to apply the iodination technique to the post-column enhancement of the fluorescence of AFB_1 and AFG_1 separated by reversed-phase liquid chromatography. They introduced iodine in the flow stream of the mobile phase by means of a T-mixer, the reaction taking place at high temperatures (60°C). The fluorescent emission of AFB_1 and AFG_1 was enhanced by a factor of 50 and no effect was observed on AFB_2 and AFG_2. Subsequently, Tuinstra and Haasnoot [137] used this method for the analysis of aflatoxins in feedstuffs. In 1984, Shepherd and Gilbert [133], carried out a series of systematic investigations for the optimization of the reaction conditions (coil tubing dimensions, coil temperature, eluent and reagent concentration, etc.). The maximum intensity of fluorescence was reached with saturated iodine solutions, in short reaction times (3–5 s) and using high temperatures (75°C). The different limits of detection (LODs) reached by this method were 0, 1, 2 ppb depending of the performance detector used. This technique has been applied to the analysis of a variety of commodities [44,47,53,54,138–140]. This method has been applied also to the analysis of feedstuff containing citrus pulp [140] yielding a good resolution of aflatoxins with no interfering peaks [128]. The derivatization method using an iodine post-column was finally adopted as an official method by the AOAC–IUPAC [141]. Although good results have been reported, halogenation system presents several drawbacks: extended time to stabilize the mobile phase (at least 1 h) heating on 75°C, dilution caused by reagent addition, the iodine solution must be prepared daily for stability reasons, an additional pump is needed and the use of very saturated solutions contributes to the great physical and mechanic deterioration of the connection tubing and the post-column pumping device suffers, owing to its prolonged contact with iodine. An alternative

way to add iodine to the solution was developed in 1987 by Jansen et al. [142] using a small column packed with solid iodine as a solid-phase reactor.

Kok et al. [143] proposed a post-column method which used derivatization with bromine as reagent. The bromine is a stronger oxidizer than iodine, but it is also less stable, so that an electrochemical generation system (KOBRA cell) must be used [144]. The reaction is complete in 4 s at room temperature, reaching similar detection limits to those reported for the iodine systems where a reduction of interfering peaks was observed. The bromine post-column derivatization was also profusely used in a variety of matrices including urine [100], air-borne dust [145], medicinal herbs and plant extracts [57] as well as foods [146,147]. The bromine derivatization was also used in HPLC with a flow injection sub-system as a post-column reactor–detector [124]. In 1993, a new method of post-column derivatization was developed using pyridinium bromide perbromide (PBPB) which provides a stable solution that needs no long reaction times or elevated temperatures. This system also has the advantage of not requiring expensive equipment such as the method using electrochemically generated bromine [56].

An alternative way explored by us to avoid all these drawbacks, allowing at the same time an enhanced fluorescent response of AFB_1 and AFG_1, is the use of cyclodextrins (CDs). CDs are oligoholosides with a chiral–toroidal configuration, yielded by the enzyme CD-transglycolase from dextrans. These cyclic oligomers contain from six to eight glucose units linked by an α(1–4) bond. Several kinds of CDs are commercially available (including α-, β-, γ-hydroxypropyl and heptakis-2,6-*ortho*-dimethyl-β-CD), and their physical and chemical properties are well described in scientific literature [148].

These oligomers have the ability to include a large number of organic and inorganic species in their cavity. Francis et al. [35] studying the interactions between β-CD and aflatoxins in purification systems, observed that the complexes formed with AFB_1 and AFG_1 showed a strong increase in the intensity of fluorescence. They also checked that when adding a post-column β-cyclodextrin solution in to a RP-HPLC system with fluorescence detection, sensitivity for detection of AFB_1 and AFG_1 was higher. However, there was no reference made above about the spectroscopic characteristics of the AF–CD inclusion complex. The spectroscopic interactions between CD and coumarin derivatives (which exhibit similar structure to AF) have been studied, especially for their subsequent application as a detection method in RP-HPLC [149]. Based on the similarity between the coumarin derivatives and aflatoxins, the spectroscopic support was described for the interaction of several CDs with aflatoxins of interest [150]. In this study, the authors demonstrated that a high CD level is necessary to demonstrate the aflatoxin fluorescence exhaltation effect. Using a concentration of 1×10^{-2} *M*, the heptakis-2,6-*ortho*-dimethyl-β-CD (DM-β-CD) exhibited a fluorescent signal two to three times higher than β-CD, eight times higher than by α-CD and 16 times larger than by γ-CD (that did not show any apparent effect). Vázquez et al. [151] later carried out a thermodynamic study in order to get an insight into the parameters that govern the interactions of AF–CD, in order to evaluate if CDs can act as versatile fluorophores for their postcolumn use in a RP-HPLC system. In this work, a mobile phase containing methanol and a high CD concentration was used, which indicates that only partial inclusion of aflatoxins takes place since methanol molecules acts as competitors for the CD cavity.

This previous knowledge was applied for the comparative study of the effect of different cyclodextrins on the aflatoxins fluorescence and to develop an analytical method based on the incorporation post-column of a CD solution that promotes the greatest enhancement of AFB_1 and AFG_1 fluorescence (Fig. 2). This enhancement was comparable to that produced by halogenation but without the disadvantages especially in reagent instability and erosion of the pumping device [152].

Subsequently, Franco et al. [153] studied the interaction between CDs and the aflatoxins Q_1, M_1 and P_1 and a substantial enhancement of AFQ_1 fluorescence in the presence of aqueous solutions of α-, β-, hydroxypropyl-β- and dimethyl-β CD was observed. On the contrary, no important fluorescence enhancement was found for AFP_1 and AFM_1 for any of the CDs tested. Vazquez et al. [154] developed a RP-HPLC system using post-column derivatization with dimethyl-β CD in order to enhance the signals of AFQ_1, AFP_1 and AFB_1 allowing urine determination in patients.

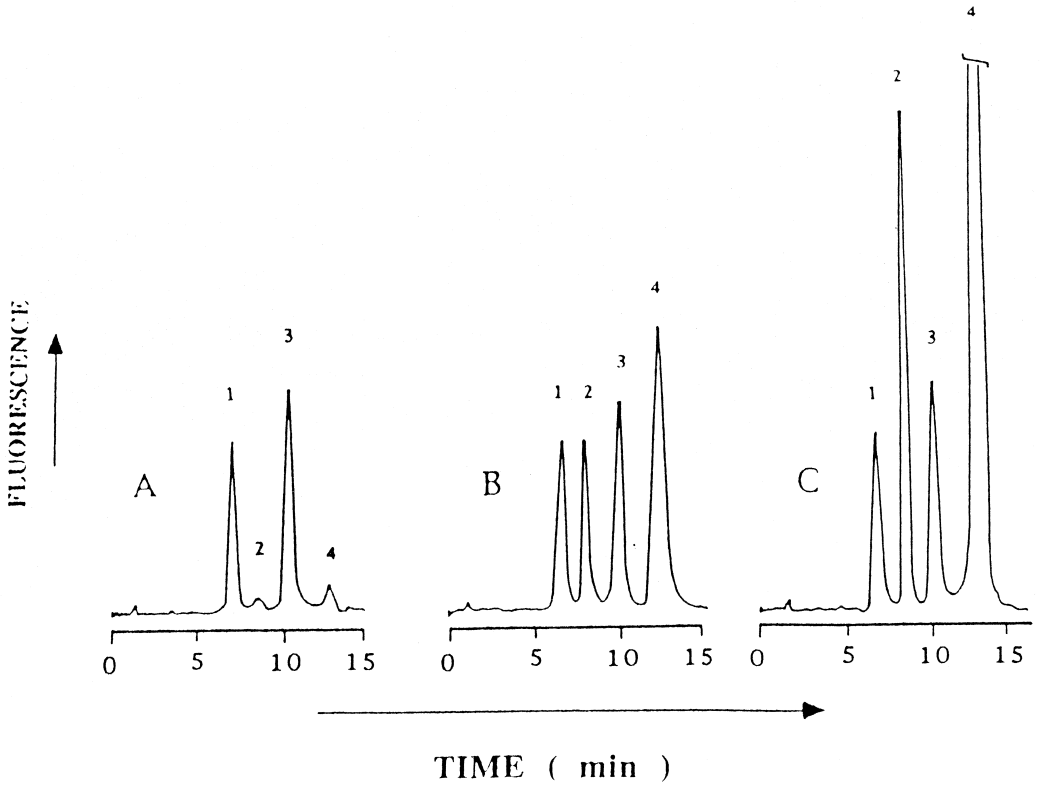

Fig. 2. Comparison of the different chromatograms: (A) without CD; (B) with the addition of 10^{-2} *M* β-CD; (C) with the addition of 10^{-2} *M* DM-β-CD. (1) AFG2; (2) AFG1; (3) AFB2; (4) AFB1.

Finally, whatever the effects performed in order to increase the fluorescence emission of most of the aflatoxin derivatives, it should be emphasized that the wide acceptance of the HPLC-fluorescence detection mode is strongly dependent on the technological performance of the fluorometric detector used (high out-put of the xenon), limited stray lie, low volume cell and reduced void volume.

Finally, the stand-point in comparison to other analytical methods especially immunochemical methods, is unsurpassed selectivity due to either the chromatographic separation and for fluorescence emission similar to the LOD detection range i.e.: ng/g of material.

3. Conclusions

Although by using NP-HPLC, the four major aflatoxins (G_1, G_2, B_1, B_2) can be easily separated in food and feedstuff, reversed-phase methods are now largely preferred. For RP-HPLC, three detection modes are widely used to enhance the least fluorescent AFB_1 and AFG_1: precolumn derivatization with TFA and post-column derivatization with iodine or bromine, the results obtained with these three methods are comparable, the fluorescence intensities for AFB_1 and G_1 are increased to the level of AFB_2 and AFG_2 in aqueous solution. TFA is the most simple method but its use is limited due to the instability of the B_{2a} and G_{2a} compounds and its use introduces an additional step to the analysis. Post-column enhancement of fluorescence is desirable because it offers an easily controlled on-line step. A post-column derivatization method using iodine presents many experimental problems which are minimized when electrochemically generated bromine is used instead of iodine, however, this method seems to have a reduced acceptance. The present needs for HPLC fluorescence detection of aflatoxins determination in

food and feedstuffs are an emphasis on the improvement of the sampling and extraction steps to lead to more accurate determinations, and further investigations of non-destructive post-column derivatization methods such as, for instance, the use of cyclodextrin inclusion compounds. An equivalent method appears to be a large unexplored field.

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