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## Review

# Derivatization reactions for the determination of aflatoxins by liquid chromatography with fluorescence detection

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### Abstract

Various derivatization methods for the fluorometric detection of aflatoxins after separation by HPLC are reviewed. In normal-phase chromatography the sensitivity for aflatoxins B<sub>1</sub> and B<sub>2</sub> was improved by using special mobile phases or a flow cell packed with silica-gel particles. In the nowadays more popular reversed-phase methods, the fluorescence intensity of B<sub>1</sub> and G<sub>1</sub> can be increased by precolumn derivatization with trifluoroacetic acid or by postcolumn derivatization with iodine or bromine. Optimum conditions for the reactions are discussed. In terms of sensitivity, the three derivatization schemes give similar results. The methods are compared with respect to experimental convenience, selectivity, reproducibility and suitability for automation.

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### 1. Introduction

Aflatoxins are a group of mycotoxins produced as secondary metabolites by fungi, mainly by *Aspergillus flavus* and *Aspergillus parasiticus*, but to a smaller extent also by other strains [1]. Aflatoxins can be produced on crops in the field or during storage of agricultural products, especially under warm and moist conditions. The

discovery of aflatoxins followed upon the appearance of the turkey "X" disease in the UK in the early 1960s [2]. In one of the feed components (peanut meal) fluorescent compounds were found which appeared to be highly toxic. Later, these compounds were identified and named aflatoxins. Although a large number of aflatoxins exist [3], only a limited number is important in (analytical) practice. Aflatoxin B<sub>1</sub> (see Fig. 1) is

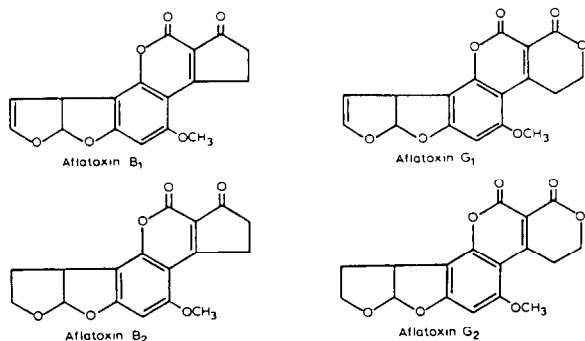


Fig. 1. Structures of the main aflatoxins.

the most widespread found in food and feed products such as peanuts, corn and cottonseed. It is highly toxic and a suspected carcinogen. The aflatoxins B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub> are usually found accompanying B<sub>1</sub> in lower concentrations in the contaminated samples. Aflatoxin M<sub>1</sub> is found in meat, eggs and milk from cattle fed with aflatoxin containing feed.

In most countries some legislation exists on the control of aflatoxins in food and feed. This regulation often includes tolerances in different products, prescribed methods of sampling and analysis and rules for the deposition of contaminated commodities. A survey of the legal situation in a large number of countries in 1987 was carried out by Van Egmond [4]. Tolerances for, e.g. peanut products are sometimes given for aflatoxin B<sub>1</sub> and sometimes for the sum of B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub>. For B<sub>1</sub> the tolerated concentration in foodstuffs is usually 5 µg/kg, while for the total concentration values of 10 or 20 µg/kg are often given. In a smaller number of countries the aflatoxin M<sub>1</sub> content of dairy products is also regulated. Tolerances of 0.05 or 0.5 µg/kg are often employed.

Because of the serious health risks of mycotoxins and the subsequent legislation, the design and improvement of methods of analysis for aflatoxins has been a major concern in analytical chemistry in the past 25 years. Governmental institutions and health protection agencies apply these methods on a large scale to control marketed food products and animal feed. In the food processing industry the same methods are used to check raw materials and products, in order to

direct them to countries with an appropriate legislation.

In several recent publications [5–8] an overview is given of the developments in aflatoxin analysis. When the total aflatoxin concentration is to be determined, batch fluorometry with the addition of a bromine solution (the SFB method) can be applied after appropriate sample clean-up [9]. When however a speciation of the aflatoxins is required, chromatographic methods are used. While in the beginning thin-layer chromatography (TLC) was the standard method of separation [10], high-performance liquid chromatography (HPLC) is now generally accepted.

In general it can be stated that at present not the determination itself but the sampling and the sample clean-up steps are the problematic parts of the analytical procedure. Concerning the sampling for instance, it was shown in a survey by Gilbert and Shepherd [11] that in peanut and brazil commodities the aflatoxin contamination was sometimes located on a few individual nuts containing extremely high aflatoxin levels. The contaminated nuts could even be identified on the basis of visible mould or discoloration.

The sample clean-up procedures employed in laboratories include liquid–liquid extraction, solid-phase extraction, column liquid chromatography and/or thin-layer chromatographic steps. The sample clean-up is in practice the most laborious and time consuming and the source of a large part of the experimental error. Therefore, much effort is still devoted to the improvement of these procedures. A recent development is the use of immunoaffinity columns for sample clean-up [9,12,13].

Even though the separation of aflatoxins by HPLC is relatively simple, optimization of the separation and detection conditions is still of importance, since it may ease the requirements for the sample clean-up. In the first successful attempts to use HPLC for aflatoxin analysis [14–16], normal-phase chromatography was employed with a UV detector set at 360–365 nm. Detection limits in the order of 1–10 ng were obtained, which would be inadequate for determinations in the concentration range of the present legal limits set for foodstuffs. It was soon

recognized that with fluorescence detection, using an excitation wavelength around 360 nm and emission at  $>420$  nm, much lower aflatoxin concentrations could be measured. However, the problem with fluorescence detection is that the sensitivities for the four major aflatoxins ( $B_1$ ,  $B_2$ ,  $G_1$  and  $G_2$ ) in solution strongly depend on the composition of the solvent. For instance, in the chloroform or dichloromethane containing mobile phases originally used for normal-phase chromatography, the aflatoxins  $B_1$  and  $B_2$  hardly show fluorescence. This made it necessary to use a UV detector (for  $B_1$  and  $B_2$ ) and a fluorescence detector (for  $G_1$  and  $G_2$ ) simultaneously [17]. On the other hand, in the aqueous solvents used in reversed-phase chromatography, the fluorescence of the  $B_1$  and  $G_1$  species is strongly diminished.

Over the years several strategies have been developed to make fluorescence detection possible for all four major aflatoxins. These strategies include an adaptation of the mobile phase composition or the detection cell in normal-phase chromatography, and pre- and post-column derivatization schemes in reversed-phase chromatography. In this review these strategies will be discussed and compared. The effect of the various methods on the detection of aflatoxin  $M_1$  and other compounds in multimycotoxin analysis will also be discussed shortly.

## 2. Fluorescence detection in normal-phase chromatography

Remarkable differences in fluorescence intensities between individual aflatoxins are found, depending on the composition of the solvent. Chang-Yen *et al.* [18] studied the fluorescence of aflatoxins in chloroform–methanol mixtures. In pure chloroform the maximum fluorescence intensities of  $B_1$  and  $B_2$  are one to two orders of magnitude lower than those of  $G_1$  and  $G_2$ . When the polarity of the solvent was increased by adding methanol, the fluorescence of  $B_1$  first increased, but at methanol concentrations higher than 50% (v/v) it decreased again. The fluorescence of  $B_2$  increased steadily with increasing

methanol concentration, that of  $G_1$  decreased, while the fluorescence of  $G_2$  was unchanged.

The influence of the solvent had already been studied by Manabe *et al.* [19], who looked for a suitable mobile phase for liquid chromatography. The key factor to increase the fluorescence of  $B_1$  and  $B_2$  was the addition of formic acid to the solvent. In pure toluene this also increased the signals for  $G_1$  and  $G_2$ ; in a mixture of toluene, ethyl acetate and methanol the addition of acid was not necessary to obtain high  $G_1$  and  $G_2$  signals. For the separation of the aflatoxins on a silica-gel column Manabe *et al.* [19] proposed a mobile phase of toluene–ethyl acetate–methanol–formic acid (89.0:7.5:1.5:2.0, v/v). Compared to the use of chloroform–dichloromethane as mobile phase, the sensitivity for  $B_1$  and  $B_2$  was improved by a factor of 20. The sensitivity for  $G_1$  was reduced by 60%. Addition of an acid to a chloroform containing mobile phase was not tried. However, it had been shown before [20] that with a chloroform–acetic acid mobile phase the sensitivity for  $B_1$  and  $B_2$  was approximately one order of magnitude lower than for  $G_1$  and  $G_2$ .

The mobile phase proposed by Manabe *et al.* [19] has been used by several others with minor modifications. Howell and Taylor [21] used the method to analyse mixed animal feeds and found detection limits of approximately 0.2 ng, corresponding to 0.4  $\mu\text{g}/\text{kg}$  in the feed samples. Goto *et al.* [22] showed that the method could be applied also for the determination of aflatoxins  $M_1$  and  $M_2$  in milk and milk products, with a limit of determination of approximately 0.1  $\mu\text{g}/\text{kg}$ . Leitao *et al.* [1] measured the aflatoxin production of various *Aspergillus* strains in this way.

A different approach to improve the fluorescence of the  $B_1$  and  $B_2$  species in normal-phase chromatography was introduced by Zimmerli [23] and by Panalaks and Scott [24] in 1977. It had been noted that the fluorescence of  $B_1$  and  $B_2$  in the adsorbed state, for instance on a TLC plate, is much stronger than in chloroform solution [25,26]. Following a strategy which had been successful for other organic compounds [27], the flow cell of the fluorometric detector was filled

with macroparticulate (0.1–0.2 mm) silica-gel, on which the eluting aflatoxins were reversibly adsorbed in a post-column reaction. Only a small increase of the peak widths was observed compared to a system with an empty cell. Sensitivities for  $B_1$  and  $B_2$  were improved to the level of the  $G_1$  and  $G_2$  species in various organic solvent mixtures. The use of a silica-gel-packed flow cell also improved the sensitivity for  $B_1$  and  $G_1$  in water–methanol mixtures used as mobile phase in reversed-phase chromatography [25], but their fluorescence intensity remained below that of the  $B_2$  and  $G_2$  species.

The packed flow cell method was used in aflatoxin analysis around 1980. A prepacked flow cell could be obtained commercially. Knutti *et al.* [28] used it in reversed-phase chromatography for the analysis of peanuts. With normal-phase chromatography the packed cell was applied for the analysis of corn [29], spices [30], peanut butter [31], and maize [32]. It has also been used for the determination of aflatoxin  $M_1$  and  $M_2$  in milk products [32,33].

At present normal-phase HPLC is not often used anymore for aflatoxin determinations. As has been the trend for HPLC in general, it has largely been replaced by reversed-phase methods. In aqueous solvents the fluorescence of the  $B_1$  and  $G_1$  species is diminished, and derivatization schemes are required for these compounds.

### 3. Precolumn hydrolysis of $B_1$ and $G_1$

The double bonds in the dihydrofuran moieties of the aflatoxins  $B_1$  and  $G_1$  (Fig. 1) are readily hydrated in acidic solution, with the production of the species  $B_{2a}$  and  $G_{2a}$ , respectively. Since these species exhibit a fluorescence intensity comparable to that of  $B_2$  and  $G_2$  in aqueous solution, this reaction has been employed for precolumn derivatization in reversed-phase chromatography. Pons *et al.* [34] have studied the kinetics of the reaction in acidified aqueous solutions. The results of this study were:

- the rate of the reaction is proportional to the  $H^+$ -concentration in the solution;
- the reaction rate increases with a factor of

approximately 1.8 for every  $10^\circ C$  temperature increase;

- the rate constant for  $B_1$  is approximately 50% higher than that for  $G_1$ .

Typical reaction times for 95% conversion at pH 1 are from 3 h at  $40^\circ C$  to 10 min at  $100^\circ C$ . The rate of the reaction was measured by monitoring the disappearance of  $B_1$  and  $G_1$ , since the stability of the  $B_{2a}$  and  $G_{2a}$  species in the acidic solution was insufficient. The derivatives degraded to non-fluorescent compounds during the experiments.

Takahashi [35] based a derivatization scheme on the hydration reaction. After extraction and clean-up by column chromatography of (spiked) wine samples, the solvent of the extracts was evaporated and trifluoroacetic acid (TFA) was added to the residues. After a few seconds to allow for the reaction to proceed, a 10% acetonitril solution was added which could be injected on a reversed-phase column. The conversion of  $B_1$  and  $G_1$  was complete, while the  $B_2$  and  $G_2$  compounds were unaffected. In Fig. 2 the effect of the hydrolysis on the separation and fluorescence intensity of aflatoxin standards is shown. The detection limits were 0.5 ng for all 4 aflatoxins, corresponding to a concentration in the wine samples of 0.02  $\mu g/l$ . The superiority over UV detection at 345 nm is clear from the figure.

Beebe [36] adapted Takahashi's method for use with various foodstuffs. To liberate the aflatoxins from the waxy residues remaining after sample extraction and clean-up, hexane was added first and then TFA. The hexane layer did not interfere with the TFA reaction.

Although it was shown that other acids such as concentrated hydrochloric acid can be used as well for the hydration reaction [37,38], the TFA method has been adopted as standard method [10]. The TFA method has been used for a variety of matrix materials. When pure TFA is added to a dry residue, the reaction of  $B_1$  and  $G_1$  is complete within 30 s at room temperature [39]. When however the TFA is added as an aqueous solution, longer times and an elevated temperature may be required. Haghighi *et al.* [40] heated a TFA–water mixture added to the residue for

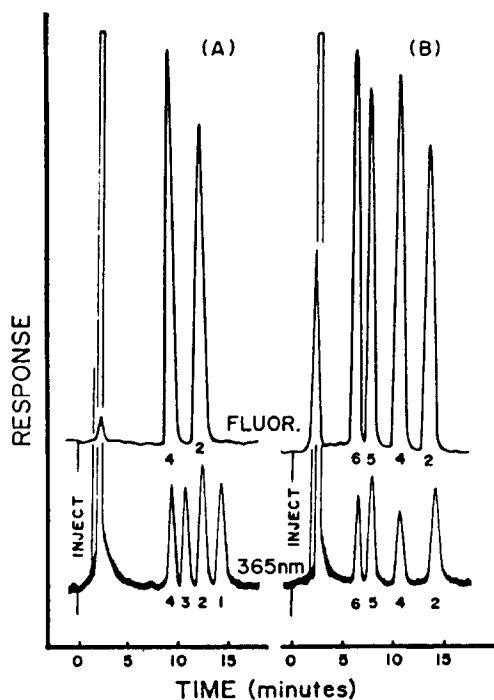


Fig. 2. Reversed-phase chromatograms of aflatoxins (25 ng each). (A) Unhydrated; (B) hydrated. Peaks: 1 = Aflatoxin B<sub>1</sub>; 2 = B<sub>2</sub>; 3 = G<sub>1</sub>; 4 = G<sub>2</sub>; 5 = B<sub>2a</sub>; 6 = G<sub>2a</sub>. Reproduced from ref. 35 with permission.

30 min at 50°C. According to Wilson and Romer [41] the reaction time could be shortened to 8.5 min by increasing the temperature to 65°C.

With the TFA method the sensitivity for B<sub>1</sub> and G<sub>1</sub> becomes similar to that for B<sub>2</sub> and G<sub>2</sub>. Although the retention of the compounds in reversed-phase systems changes by the derivatization, generally the required adaptation of the chromatographic conditions is negligible. Sometimes the TFA has to be evaporated after the reaction, before the injection solution is added, to avoid excessive peak broadening on the first part of the column [42]. The reproducibility for the derivatized B<sub>1</sub> and G<sub>1</sub> compounds is comparable to that for B<sub>2</sub> and G<sub>2</sub>, which indicates that the derivatization procedure does not introduce significant error. However, according to Tarter *et al.* [43] this is only true when the reaction is allowed to proceed for a sufficiently long time (5 min).

The main disadvantage of the TFA method is

the limited stability of the B<sub>2a</sub> and G<sub>2a</sub> compounds. As has been shown by Beaver [44], the stability of underivatized aflatoxins in common HPLC solvents can already be a problem, especially when the solutions are exposed to light. However, by addition of acetic acid to the solutions the degradation of these compounds can be diminished. The degradation of B<sub>2a</sub> and G<sub>2a</sub> is less easy to prevent; methanol should be avoided in the solutions in any case [7]. The stability problems may be important when large numbers of samples are analyzed, and the solutions after clean-up and derivatization have to be kept for a long time in the autosampler before the actual analysis takes place.

#### 4. Post-column derivatization with iodine

Davis and Diener [45] noticed that the fluorescence intensity of aflatoxin solutions increased after the addition of iodine. They used this reaction in reversed-phase HPLC [46]. When iodine was added to a solution of B<sub>1</sub> before injection, the B<sub>1</sub> peak disappeared from the chromatogram and a new peak with a 25-fold larger intensity appeared. A similar behaviour was observed for G<sub>1</sub>. They used this method only as a confirmatory test; the reaction could not be used as a standard derivatization procedure since multiple secondary products were formed. Later it was shown by thermospray mass spectrometry that the main product of the iodine reaction in water–methanol mixtures is a compound with one iodine atom and one methoxy-group added to the double bond of the B<sub>1</sub> and G<sub>1</sub> compounds [47].

The formation of multiple products is not a hindrance in post-column reaction methods. This was first recognized by Thorpe *et al.* [48] and later utilized by Tuinstra and Haasnoot [49] for the development of a post-column derivatization method for aflatoxins B<sub>1</sub> and G<sub>1</sub> in reversed-phase chromatography. A iodine solution is added through a T-piece to the column effluent, the mixture is pumped through a stainless steel or PTFE reaction coil kept at elevated temperature to allow for the reaction to proceed, and the

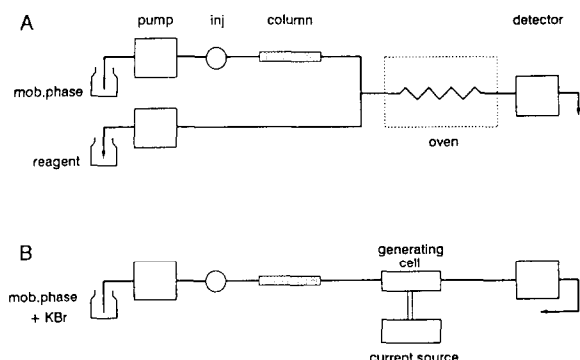


Fig. 3. Schemes of the set-up for post-column derivatization of aflatoxins (A) with a saturated iodine solution, (B) with electrochemically generated bromine.

reaction products are monitored with a fluorescence detector (see Fig. 3A). The sensitivity for  $B_1$  and  $G_1$  is improved by the derivatization with a factor of 25–50. Shephard and Gilbert [50] conducted a systematic investigation on the optimization of the reaction conditions, such as the mobile phase and reagent flow-rates, the coil dimensions, the temperature and the reagent concentration. They found that the fluorescence intensities for  $B_1$  and  $G_1$  increased with the concentration of the reagent solution, giving the highest peaks when a saturated iodine solution was used. The use of a saturated aqueous iodine solution as reagent has become common practice. This, however, is a cause of experimental difficulties. The saturated reagent has to be

prepared fresh daily by filtering out an excess of solid iodine, and clogging of capillaries and wear of the seals of the reagent pump may occur.

Depending on the temperature of the reaction coil, the required reaction time can be relatively long; reaction times up to 2 min have been used. The use of wide and/or long reaction coils may cause appreciable extra-column peak broadening. Different reaction coil geometries and reaction temperatures have been used. In Table 1 examples are given of the experimental conditions as reported in the literature. It is obvious that a short reaction time can be used when a high coil temperature is applied. With reported reaction times of 3–5 s, extra-column peak broadening should not be a problem.

In all references the sensitivity for  $B_1$  and  $G_1$  increases to the level of the  $B_2$  and  $G_2$  compounds by the derivatization. Differences in reported detection limits (from 2 to 100 pg injected) and limits of determination in food and feed samples (from 0.1 to 2  $\mu\text{g}/\text{kg}$ ), should be attributed to the quality of the fluorescence detectors employed and the efficiency of the clean-up procedures. The iodine derivatization method has been adopted as an official AOAC–IUPAC method [58].

An alternative way to add iodine to the solution has been developed by Jansen *et al.* [59]. They used a small column packed with solid iodine as a solid-phase reactor. By splitting before the injector, a small part of the mobile-

Table 1  
Experimental conditions for post-column derivatization with iodine

Year	Temperature (°C)	Coil length (m)	Diameter (mm)	Reaction time (s)	Ref.
1983	60	3	0.5	40	49
1984	75	5	0.3	17	50
1985	75	5	0.3	16	11
1986	60	10.7	0.34	67	51
1987	62	15	0.5	106	52
1988	60	3	0.5	40	53
1989	90	0.25	0.5	3	54
1990	75	3	0.25	5	55
1991	70	6.1	0.5	55	9
1991	68	5	0.3	17	56
1991	70	5	0.3	14	13,57

phase flow was led through the solid-phase reactor and in this way saturated with iodine. The reagent flow was combined again with the column effluent to react with the eluting aflatoxins. The advantages of this set-up are that only one pump is required and that the dilution of the column effluent with reagent solution is minimal.

### 5. Other post-column derivatization methods

The increase in sensitivity for  $B_1$  and  $G_1$  in aqueous solutions can be brought about by oxidizing reagents other than iodine, such as bromine. Since bromine is a stronger oxidator than iodine, it may be expected that its reaction rate with aflatoxins is higher and that a lower reagent concentration can be used. This has been investigated by Kok *et al.* [60,61], who devised a post-column derivatization method with bromine. Since bromine in solution is even less stable than iodine, the reagent was not added as a solution with a second pump. Instead, bromide was electrochemically generated on-line from a bromide salt added to the acidified mobile phase (see Fig. 3B). After the column the eluent is passed through an electrochemical cell (the KOBRA cell [62]), where bromine is produced by a constant oxidative current. The rate of bromine production, and with that the concentration of bromine in the column effluent, is controlled by the height of the current. After a short reaction coil the solution is led through the fluorescence detector. The outlet of the detector is connected to the counter-electrode compartment of the KOBRA-cell, to sweep away reaction products of this electrode. A similar set-up had been used for the post-column oxidation of phenothiazines [63].

It was shown that the reaction of  $B_1$  and  $G_1$  was complete within 4 s at room temperature. Optimal generating currents were between 50 and 100  $\mu\text{A}$ , corresponding to reagent concentrations of 3 to  $6 \cdot 10^{-5} \text{ mol l}^{-1}$ . At higher currents the peak heights for all aflatoxins decreased, probably by oxidative degradation of the compounds. Similar effects of the reagent

concentration have been observed by others in a flow-injection system [64]. The sensitivities obtained for the derivatized  $B_1$  and  $G_1$  compounds was approximately equal to that with a iodine derivatization system. For the non-derivatized  $B_2$  and  $G_2$  the sensitivity was two times higher; this can be explained by the fact that the column effluent is not diluted here. The detection limits were in the order of 20–40 pg injected. The system has been used for the determination of aflatoxins in cattle feed; results were in good agreement with other methods. As advantages of the bromine system over the iodine system have been mentioned:

- the equipment is less expensive, since only one pump is required;
- the installation, operation and maintenance are easier; the daily preparation of the saturated iodine solution is avoided;
- with citrus fruit containing samples, the method is more selective; interfering citrus peaks are absent or less intense.

Fig. 4 shows the chromatograms obtained with a (fortified) cattle feed extract using the iodine and the bromine system. The reduction of interferences from the matrix by bromine derivatization has also been observed by others [65]. The method has been validated in ring-tests of the European Community [66]. The experimental parameters have been optimized further by Kusak *et al.* [67] with the help of factorial design.

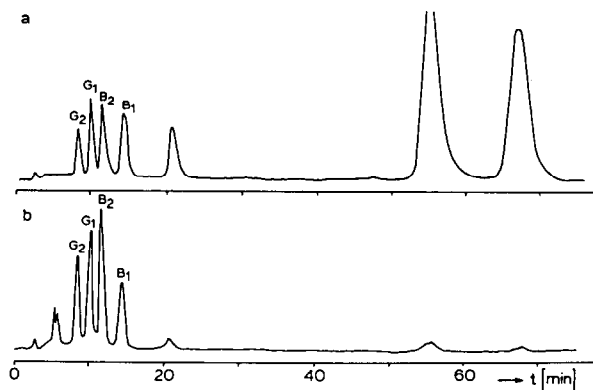


Fig. 4. Chromatograms obtained with (fortified) cattle feed extracts. Post-column derivatization with (a) iodine; (b) bromine. Reproduced from ref. 60.

When Francis *et al.* [68] studied the interaction between aflatoxins and  $\beta$ -cyclodextrins, in the search for simplified clean-up procedures, they found that the complexes of B<sub>1</sub> and G<sub>1</sub> with  $\beta$ -cyclodextrin showed a strongly increased fluorescence intensity. By adding an aqueous  $\beta$ -cyclodextrin solution post-column, they could improve the detection sensitivity for these compounds to that of B<sub>2</sub> and G<sub>2</sub>. Later spectroscopic studies [69] showed that  $\alpha$ -cyclodextrins have the same effect, while  $\gamma$ -cyclodextrins are not effective.

## 6. Detection of other mycotoxins

For the determination of aflatoxins M<sub>1</sub> and M<sub>2</sub> in milk and other dairy products, the chromatographic systems developed for the B and G aflatoxins have been used, sometimes with slight modifications. In normal-phase chromatography with an acidified toluene-containing mobile phase, the detection limits for M<sub>1</sub> and M<sub>2</sub> are approximately 5 times higher than for the other aflatoxins [22]. Silica-gel packed flow cells have also been used [32,33]. For reversed-phase HPLC, TFA derivatization increases the fluorescence 3 to 4 times when M<sub>1</sub> is converted to a compound designated as M<sub>2a</sub> [40]. Cohen *et al.* [70] used the TFA reaction for the confirmation of the presence of M<sub>1</sub> in milk. Purified sample extracts were divided in two parts, one of which was treated with TFA. This resulted in the disappearance of the M<sub>1</sub> peak from the chromatogram. The limit of detection for the underivatized M<sub>1</sub> was approximately 5 pg injected. The M<sub>2a</sub> peak could not be quantified since it coeluted with matrix interferences.

Hisada *et al.* [71] used a modified TFA method for the determination of M<sub>1</sub> in cheese. They found that the presence of hexane during the TFA reaction prevented the appearance of a by-product. The conversion to M<sub>2a</sub> was complete in 20 min at 40°C. The sensitivity for M<sub>2a</sub> was approximately four times higher than that for M<sub>1</sub>. A further confirmation of the identity of the M<sub>2a</sub> peak could be obtained by a second derivatization reaction. After the first derivatiza-

tion step, the M<sub>2a</sub> containing solutions were evaporated to dryness, methanol and TFA were added, and the test tube was heated for another 10 min at 40°C. This reaction caused the disappearance of the M<sub>2a</sub> peak and the appearance of a new peak later in the chromatogram.

Since the gain in fluorescence intensity for M<sub>1</sub> is not as large as for B<sub>1</sub> and G<sub>1</sub>, post-column derivatization methods have not been developed.

Recently there is a widespread interest in multi-mycotoxin analyses. By combined clean-up steps and separation procedures, more economic methods of analysis can be obtained. The mycotoxins most often determined simultaneously with aflatoxins are ochratoxin and zearalenone. Both compounds can be detected after HPLC separation on the basis of their native fluorescence [20, 72–76]. Chamkasem *et al.* [54] developed an integrated system for the simultaneous determination of aflatoxins, ochratoxin and zearalenone. They used two fluorescence detectors. The first, set at excitation and emission wavelengths of 236 and 419 nm, respectively, was directly connected to the separation column. The second detector was connected after a T-piece, where an iodine solution was added, and a heated reaction coil. The first detector was used for the quantification of ochratoxin and zearalenone, the second for the aflatoxins. By the reaction with iodine the zearalenone peak disappeared completely, while the height of the ochratoxin peak decreased strongly. The (partial) absence of these peaks in the second chromatogram was used for the confirmation of the signal from the first detector.

An alternative method for this multi-toxin determination was developed by Dunne *et al.* [65]. They used only one fluorescence detector and switched the wavelengths during the chromatographic run. For the detection of aflatoxins post-column derivatization with bromine was used. While ochratoxin was not affected by the bromine derivatization, the signal for zearalenone disappeared. With a second run without derivatization, zearalenone was measured. By comparison of the chromatograms with and without derivatization, the identities of aflatoxin B<sub>1</sub> and G<sub>1</sub> and zearalenone could be confirmed.



## 7. Conclusions

With normal-phase chromatography the four major aflatoxins in food and feed stuffs can easily be separated. By employing suitable mobile phases or a silica-packed flow cell, a satisfactory sensitivity and selectivity has been obtained with fluorescence detection. Still, in aflatoxin analysis normal-phase chromatography is now largely replaced by reversed-phase methods. This is not because better results can be obtained with reversed-phase HPLC, but for the same general reasons (solvent costs, safety and waste disposal problems) as in many other applications of HPLC.

For reversed-phase chromatography, three methods are now widely used to improve the detectability of the B<sub>1</sub> and G<sub>1</sub> aflatoxins: pre-column derivatization with a strong acid (TFA), and postcolumn derivatization with iodine or bromine. The results of the three methods are comparable: the fluorescence intensities for B<sub>1</sub> and G<sub>1</sub> are improved to the level of the B<sub>2</sub> and G<sub>2</sub> species in aqueous solutions. Since the fluorescence quantum yields of the latter species are already high [69], further significant improvements of the derivatization methods are not to be expected.

The main advantage of the TFA method is its simplicity. When pure TFA can be added to a dry residue the reaction is complete in a few seconds at room temperature. The sample clean-up procedure should therefore result in a solution which is easily evaporated. This makes the application of modern automated clean-up procedures more problematic. In principle, the addition of an extra step to the sample preparation procedure creates an extra source of experimental error. However, this has not been confirmed in practice. The reproducibility for B<sub>1</sub> and G<sub>1</sub> is similar to that for B<sub>2</sub> and G<sub>2</sub>; also, the results with the precolumn method are not worse than with postcolumn methods. Apparently, other steps in the clean-up dominate the experimental error.

The reason most often mentioned to use a postcolumn derivatization method instead of the TFA method, is the limited stability of the TFA

derivatives. This instability becomes of importance when large numbers of samples have to be analyzed sequentially with automated chromatographic equipment. In the past, the number of samples which could be analyzed in one day was limited by the laborious manual sample handling. However, with the development of new, automated clean-up methods this situation may change.

The post-column derivatization method with iodine has amply proven its value in practice, and the method has been adopted as official AOAC-method. In the past fairly long and wide reaction coils have been used to ensure a complete reaction of the aflatoxins with iodine. This caused a deterioration of the separation of the individual aflatoxins. It has been shown that with a higher reaction temperature (75°C or higher) the reaction can be complete in a few seconds, so that a short and narrow coil is sufficient.

Reported experimental difficulties are related to the use of a saturated iodine solution. These solutions have to be prepared frequently, since their stability is insufficient. Also, the use of saturated solutions in pumps, connectors and narrow coils is normally not recommended. The question arises whether it is really necessary to use a saturated solution. With the postcolumn addition of the reagent to the column effluent, the reagent is diluted anyway. Moreover, although it has been reported that the yield of the reaction increases with the reagent concentration, the gain over a non-saturated solution was not spectacular. The final reaction mixture contains a certain fraction of organic solvent from the mobile phase. It may be worthwhile to study whether the reagent can be prepared by dilution of a more concentrated iodine stock solution in a water-miscible organic solvent.

Many of the experimental problems with the iodine method are circumvented when electrochemically generated bromine is used as the reagent. The required equipment is less expensive and easier to maintain. The additional selectivity of the bromine system (many fluorescent interferences are degraded in the reaction with bromine) may simplify the sample clean-up. Because of its experimental simplicity, the

bromine derivatization system has become a routine method widely applied in the Netherlands. A reason for its slow acceptance elsewhere may be that the electrochemical cell to generate bromine, although commercially available, is not widely known.

A matter of concern with post-column derivatization is that it can interfere with the fluorescence detection of other mycotoxins, especially zearalenone, in multi-toxin analysis. This could be a reason for a further investigation of the non-destructive derivatization with cyclodextrins.

In conclusion it can be stated that the emphasis on the sample preparation in aflatoxin analysis which is found in the recent literature, is justified. Detection in HPLC does not have to be a problem anymore. The compatibility of newly developed, automated clean-up procedures with the various derivatization methods should be addressed in these studies.

## References

- [1] J. Leitao, G. de Saint Blanquat, J.R. Bailly and Ch. Paillas, *J. Chromatogr.*, 435 (1988) 229.
- [2] W.O. Ellis, J.P. Smith and B.K. Simpson, *Crit. Rev. Food Sci. Nutr.*, 30 (1991) 403.
- [3] R.J. Cole and R.H. Cox, *Handbook of Toxic Fungal Metabolites*, Academic Press, New York, 1981.
- [4] H.P. van Egmond, *Food Addit. Contam.*, 6 (1989) 139.
- [5] G.E. Rottinghaus, in J.L. Richard and J.H. Thurston (Editors), *Diagnostics of Mycotoxins*, Martinus Nijhoff, Dordrecht, 1986.
- [6] V. Betina, *J. Chromatogr.*, 477 (1989) 187.
- [7] M. Holcomb, D.M. Wilson, M.W. Trucksess and H.C. Thompson, *J. Chromatogr.*, 624 (1992) 341.
- [8] J.C. Frisoad and U. Thrane, in V. Betina (Editor), *Chromatography of Mycotoxins*, J. Chromatogr. Library Vol. 54, Elsevier, Amsterdam, 1993, Ch. 8.
- [9] M.W. Trucksess, M.E. Stack, S. Nesheim, S.W. Page, R.H. Albert, Th.J. Hansen and K.F. Donahue, *J. Assoc. Off. Anal. Chem.*, 74 (1991) 81.
- [10] *Official Methods of Analysis of the Association of Official Analytical Chemists*, AOAC, Arlington, VA, 15th ed., 1990, Ch. 26.
- [11] J. Gilbert and M.J. Shepherd, *Food Addit. Contam.*, 2 (1985) 171.
- [12] M.J. Shepherd, D.N. Mortimer and J. Gilbert, *J. Assoc. Publ. Analysts*, 25 (1987) 129.
- [13] A.L. Patey, M. Sharman and J. Gilbert, *J. Assoc. Off. Anal. Chem.*, 74 (1991) 76.
- [14] R.C. Garner, *J. Chromatogr.*, 103 (1975) 186.
- [15] L.M. Seitz, *J. Chromatogr.*, 104 (1975) 81.
- [16] W.A. Pons and A.O. Franz, *J. Assoc. Off. Anal. Chem.*, 60 (1977) 89.
- [17] W.A. Pons and A.O. Franz, *J. Assoc. Off. Anal. Chem.*, 61 (1978) 793.
- [18] I. Chang-Yen, V.A. Stoute and J.B. Felmine, *J. Assoc. Off. Anal. Chem.*, 67 (1984) 306.
- [19] M. Manabe, T. Goto and S. Matsuura, *Agric. Biol. Chem.*, 42 (1978) 2003.
- [20] D.C. Hunt, A.T. Bourdon, P.J. Wild and N.T. Crosby, *J. Sci. Food Agric.*, 29 (1978) 234.
- [21] M.V. Howell and Ph.W. Taylor, *J. Assoc. Off. Anal. Chem.*, 64 (1981) 1356.
- [22] T. Goto, M. Manabe and S. Matsuura, *Agric. Biol. Chem.*, 46 (1982) 801.
- [23] B. Zimmerli, *J. Chromatogr.*, 131 (1977) 463.
- [24] T. Panalaks and P.M. Scott, *J. Assoc. Off. Anal. Chem.*, 60 (1977) 583.
- [25] B.L. van Duuren, T.L. Chan and F.M. Irani, *Anal. Chem.*, 40 (1968) 2024.
- [26] E. Sawicki, *Talanta*, 16 (1969) 1231.
- [27] J.B.F. Lloyd, *Analyst (London)*, 100 (1975) 529.
- [28] R. Knutti, Ch. Balsiger and K. Sutter, *Chromatographia*, 12 (1979) 349.
- [29] W.A. Pons, *J. Assoc. Off. Anal. Chem.*, 62 (1979) 586.
- [30] M.J. Awe and J.L. Schranz, *J. Assoc. Off. Anal. Chem.*, 64 (1981) 1377.
- [31] O.J. Francis, L.J. Lipinski, J.A. Gaul and A.D. Campbell, *J. Assoc. Off. Anal. Chem.*, 65 (1982) 672.
- [32] J. Boehm, Ch. Noonpugdee, J. Leibetseder and M. Schuh, *Ernaehrung*, 8 (1984) 675.
- [33] J. Ferguson-Foos and J.D. Warren, *J. Assoc. Off. Anal. Chem.*, 67 (1984) 1111.
- [34] W.A. Pons, A.F. Cucullu, L.S. Lee and H.J. Janssen, *J. Am. Oil Chem. Soc.*, 49 (1972) 124.
- [35] D.M. Takahashi, *J. Chromatogr.*, 131 (1977) 147.
- [36] R.M. Beebe, *J. Assoc. Off. Anal. Chem.*, 61 (1978) 1347.
- [37] G.J. Diebold and R.N. Zare, *Science*, 196 (1977) 1439.
- [38] W.J. Hurst, L.M. Lenovich and R.A. Martin, *J. Assoc. Off. Anal. Chem.*, 65 (1982) 888.
- [39] R.M. Beebe and D.M. Takahashi, *J. Agric. Food Chem.*, 28 (1980) 481.
- [40] B. Haghghi, C. Thorpe, A.E. Pohland and R. Barnett, *J. Chromatogr.*, 206 (1981) 101.
- [41] Th.J. Wilson and Th.R. Romer, *J. Assoc. Off. Anal. Chem.*, 74 (1991) 951.
- [42] O.G. Roch, G. Blunden, R.D. Coker and S. Nawaz, *Chromatographia*, 33 (1992) 208.
- [43] E.J. Tarter, J.-P. Hanchay and P.M. Scott, *J. Assoc. Off. Anal. Chem.*, 67 (1984) 597.
- [44] R.W. Beaver, *J. High Resolut. Chromatogr.* 13 (1990) 833.
- [45] N.D. Davis and U.L. Diener, *J. Appl. Biochem.*, 1 (1979) 115.

- [46] N.D. Davis and U.L. Diener, *J. Assoc. Off. Anal. Chem.*, 63 (1980) 107.
- [47] M. Holcomb, W.A. Korfmacher and H.C. Thompson, *J. Anal. Toxicol.*, 15 (1991) 289.
- [48] C.W. Thorpe, G.M. Ware and A.E. Pohland, *Proceedings of the Vth International IUPAC Symposium on Mycotoxins and Phycotoxins, Vienna, Sept. 1–3, 1982*, Vienna Technical University, p. 52.
- [49] L.G.M.Th. Tuinstra and W. Haasnoot, *J. Chromatogr.*, 282 (1983) 457.
- [50] M.J. Shepherd and J. Gilbert, *Food Addit. Contam.*, 1 (1984) 325.
- [51] P.G. Thiel, S. Stockenstrom and P.S. Gathercole, *J. Liq. Chromatogr.*, 9 (1986) 103.
- [52] W.J. Hurst, K.P. Snyder and R.A. Martin, *J. Chromatogr.*, 409 (1987) 413.
- [53] W.E. Paulsch, E.A. Sizoo and H.P. van Egmond, *J. Assoc. Off. Anal. Chem.*, 71 (1988) 957.
- [54] N. Chamkasem, W.Y. Cobb, G.W. Latimer, C. Salinas and B.A. Clement, *J. Assoc. Off. Anal. Chem.*, 72 (1989) 336.
- [55] R.W. Beaver, D.M. Wilson and M.W. Trucksess, *J. Assoc. Off. Anal. Chem.*, 73 (1990) 579.
- [56] M. Holcomb and H.C. Thompson, *J. Agric. Food Chem.*, 39 (1991) 137.
- [57] M. Sharman and J. Gilbert, *J. Chromatogr.*, 543 (1991) 220.
- [58] *Changes in Official Methods of Analysis*, Association of Official Analytical Chemists, Arlington, VA, 1991, 15th ed., Suppl. 2, Method 991.31.
- [59] H. Jansen, R. Jansen, U.A.Th. Brinkman and R.W. Frei, *Chromatographia*, 24 (1987) 555.
- [60] W.Th. Kok, Th.C.H. van Neer, W.A. Traag and L.G.M.Th. Tuinstra, *J. Chromatogr.*, 367 (1986) 231.
- [61] W.A. Traag, J.M.P. van Trijp, L.G.M.Th. Tuinstra and W.Th. Kok, *J. Chromatogr.*, 396 (1987) 389.
- [62] W.Th. Kok, U.A.Th. Brinkman and R.W. Frei, *Anal. Chim. Acta*, 162 (1984) 19.
- [63] W.Th. Kok, W.H. Voogt, U.A.Th. Brinkman and R.W. Frei, *J. Chromatogr.*, 354 (1986) 249.
- [64] F. Lazaro, M.D. Luque de Castro and M. Valcarcel, *Fresenius J. Anal. Chem.*, 332 (1988) 809.
- [65] C. Dunne, M. Meany, M. Smyth and L.G.M.Th. Tuinstra, *J. Chromatogr.*, 629 (1993) 229.
- [66] J.A. van Rhijn, J. Viveen and L.G.M.Th. Tuinstra, *J. Chromatogr.*, 592 (1992) 265.
- [67] A. Kussak, B. Andersson and K. Andersson, *J. Chromatogr.*, 616 (1993) 235.
- [68] O.J. Francis, G.P. Kirschenheuter, G.M. Ware, A.S. Carman and S.S. Kuan, *J. Assoc. Off. Anal. Chem.*, 71 (1988) 725.
- [69] M.L. Vazquez, A. Cepeda, P. Prognon, G. Mahuzier and J. Blais, *Anal. Chim. Acta*, 255 (1991) 343.
- [70] H. Cohen, M. Lapointe and J.M. Fremy, *J. Assoc. Off. Anal. Chem.*, 67 (1984) 49.
- [71] K. Hisada, H. Terada, K. Yamamoto, H. Tsubouchi and Y. Sakabe, *J. Assoc. Off. Anal. Chem.*, 67 (1984) 601.
- [72] M.V. Howell and Ph.W. Taylor, *J. Assoc. Off. Anal. Chem.*, 64 (1981) 1356.
- [73] G.A. Bennett, O.L. Shotwell and W.F. Kmolek, *J. Assoc. Off. Anal. Chem.*, 68 (1985) 958.
- [74] H. Cohen and M. Lapointe, *J. Assoc. Off. Anal. Chem.*, 69 (1986) 957.
- [75] R. Tressl, E. Hommel and B. Helak, *Monatsschr. Brauwissenschaft*, 8 (1989) 331.
- [76] M. Merino, A.J. Ramos and E. Hernandez, *Mycopathologia*, 121 (1993) 27.