FOOD CHEMICAL CONTAMINANTS

Comparison of Two Post-Column Derivatization Systems, Ultraviolet Irradiation and Electrochemical Determination, for the Liquid Chromatographic Determination of Aflatoxins in Food

ANDRI PAPADOPOULOU-BOURAOUI, JOERG STROKA, and ELKE ANKLAM[1]
European Commission, DG Joint Research Centre, Institute for Health and Consumer Protection, Food Products and Consumer Goods Unit, I-21020 Ispra, Italy

This study compared 2 post-column derivatization (PCD) techniques for the determination of aflatoxins B_1, B_2, G_1, and G_2 (AFB_1, AFB_2, AFG_1, and AFG_2) by fluorescence detection after liquid chromatographic separation: ultraviolet (UV) irradiation (PCD_{UV}) and electrochemical bromination (PCD_{EC}). Photochemical fluorescence enhancement was obtained with 2 different commercially available systems (PCD_{UV1} and PCD_{UV2}). An electrochemical bromination apparatus was used for bromination. Analyses of naturally contaminated or spiked samples of corn, pistachio paste, peanut butter, fig paste, and animal feed showed that neither of the techniques resulted in derivatization-specific matrix interferences for any of the matrixes under study, even when extracts were not completely purified. The response ratios PCD_{UV}/PCD_{EC} for AFB_1, AFB_2, AFG_1, and AFG_2 were 0.86, 0.96, 0.70, and 0.96, respectively, for PCD_{UV1} and 0.82, 0.95, 0.60, and 0.90, respectively, for PCD_{UV2}. The long-term use of the UV lamps (300 h for PCD_{UV1} and 343 h for PCD_{UV2}) in the photochemical detectors showed that these ratios remained stable throughout the time frame investigated. The relative standard deviation obtained for each of the devices during the in-house validation study ranged from 0.3 to 1.8% for PCD_{UV1}, from 0.8 to 1.3% for PCD_{UV2}, and from 0.9 to 2.0% for PCD_{EC}.

Aflatoxins B_1, B_2, G_1, and G_2 (AFB_1, AFB_2, AFG_1, and AFG_2) are toxic secondary metabolites of *Aspergillus flavus* and *Aspergillus parasiticus*, with AFB_1 being the most toxic and the most abundant aflatoxin in food (1). These aflatoxins can occur in a wide range of food commoditiies (e.g., nuts, seeds, dried fruits, and spices). The monitoring of food commodities for aflatoxins as a consequence of regulations established by >60 countries has resulted in the need for adequate analytical methods (2).

Today, aflatoxins are frequently determined by reversed-phase liquid chromatography (LC) with fluorescence detection. However, the quenching of the fluorescence of AFB_1 and AFG_1, which occurs in aqueous solvents, has led to the development of techniques allowing their transformation into nonquenchable derivatives (3). This can be achieved by either precolumn or post-column derivatization (PCD) techniques. Trifluoroacetic acid converts the double bond of the dihydrofuran moiety into hemiacetal derivatives of AFG_{2a} and AFB_{2a}, which do not undergo fluorescence quenching in aqueous solutions. Because this precolumn derivatization technique is laborious, and because of the relative instability of these derivatives and their higher polarity (4), PCD techniques have been developed and applied successfully.

One PCD technique involves post-column iodination (5–7), but it has been shown to be inferior to bromination (8). In addition, the need for daily preparation of the iodine solution, the required use of an auxiliary pump, and the lengthy preparation of the mobile phase (9) are drawbacks of this technique. Recently, fluorescence amplification of aflatoxins has also been achieved by the use of cyclodextrins (9, 10), but this technique has not been used for routine analysis thus far because it is costly and offers no advantage compared with the derivatization techniques discussed in this paper. Other widely used PCD systems are based on bromination of the aflatoxins (Figure 1). Preferably, bromine can be generated electrochemically or by the use of pyridinium hydrobromide perbromide (11–13).

In addition, post-column photolytic derivatization (PCD_{UV}) has been used successfully for the determination of a wide range of compounds (14–20), including aflatoxins (21–23). During photolysis, AFB_1 and AFG_1 are converted to hemiacetals, AFB_{2a} and AFG_{2a}, which are similar to those obtained by derivatization with trifluoroacetic acid (shown for AFB_1 in Figure 2).

The aim of the work described in this paper was the direct comparison of PCD by electrochemical bromination (Kobra cell) with PCD by ultraviolet (UV) irradiation; these techniques are currently being used in the determination of aflatoxins by LC with fluorescence detection.

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[1]Author to whom correspondence should be addressed; e-mail: elke.anklam@jrc.it.

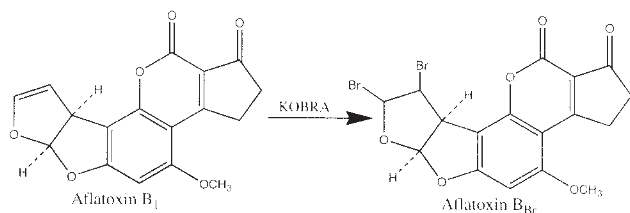

Figure 1. Schematic representation of the mechanism for the bromination of AFB_1.

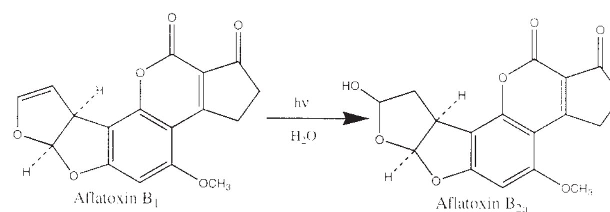

Figure 2. Schematic representation of the mechanism for the conversion of AFB_1 to AFB_{2a} by a photolysis reaction.

Experimental

Safety note: Aflatoxins are carcinogenic substances and should be handled with caution.

Reagents

(**a**) *Solvents*.—Used for extraction; American Chemical Society-International Organization for Standardization (ACS-ISO) grade; from Carlo Erba (Milan, Italy).

(**b**) *Acetonitrile and methanol*.—LC grade from Merck (Darmstadt, Germany).

(**c**) *Water*.—Passed through a Millipore water purification device (Bedford, MA).

(**d**) *Aflatoxin standards*.—Sigma-Aldrich (Milan, Italy).

(**e**) *Sodium chloride, phosphate-buffered saline, pH 7.4, potassium bromide, and nitric acid*.—Merck.

Apparatus

(**a**) *Immunoaffinity columns*.—Rhone Diagnostics Technologies Ltd. (Glasgow, UK).

(**b**) *LC system*.—Consisted of a 307 pump and an ASPEC XL solid-phase extraction (SPE) system from Gilson (Middleton, WI), a degassing unit from Jour Research (Onsala, Sweden), and a Waters 474 scanning fluorescence detector (Waters, Milford, MA). The detector was set at 365 and 435 nm for the excitation and emission wavelengths, respectively, with a bandwidth of 18 nm. The LC column was 5 µm LC-18 (25.0 × 4.6 mm) Supelcosil with precolumn (Supelco, Milan, Italy). The LC mobile phase was water–acetonitrile–methanol (6 + 2 + 3, v/v/v) containing 120 mg potassium bromide and 350 µL nitric acid (a concentration of 4M in the mobile phase).

(**c**) *Photochemical derivatization devices*.—Placed between the LC column and the detector; consisted of a lamp holder, a 254 nm low-pressure mercury lamp, and a holder for the knitted reactor coils. The reactor coils are made of polytetrafluoroethylene (PTFE) that is transparent to the 254 nm UV light. Because the coils are very long, they are literally knitted to fit around the UV lamp. System PCD_{UV1} (designated PHRED by the manufacturer) was provided by Aura Industries, Inc. (New York, NY), and system PCD_{UV2} (designated Beam Boost) was purchased from Ict Chemietechnik GmbH (Vienna, Austria). The reaction coils used were 25 and 20 m long for PCD_{UV1} and PCD_{UV2}, respectively.

(**d**) *Electrochemical bromination apparatus*.—Kobra cell; Rhone Diagnostics Technologies Ltd.; used for bromination. For the comparison, each PCD_{UV} and the PCD_{EC} (Kobra) were connected by a Rheodyne valve (Supelco) that allowed switching back and forth to each system to obtain a direct comparison between them. The setup is shown in Figure

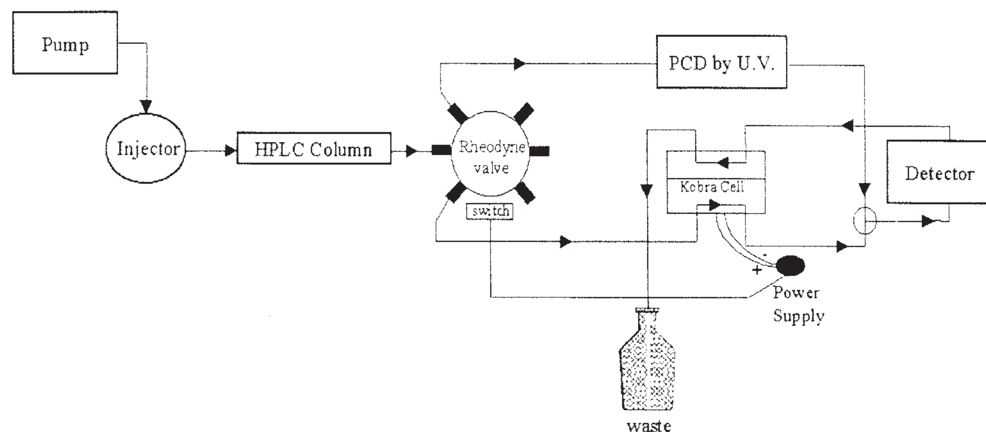

Figure 3. Schematic representation of the setup used during the direct comparison of the 2 PCD devices: UV derivatization (PCD_{UV1} or PCD_{UV2}) and electrochemical derivatization using the Kobra cell (PCD_{EC}).

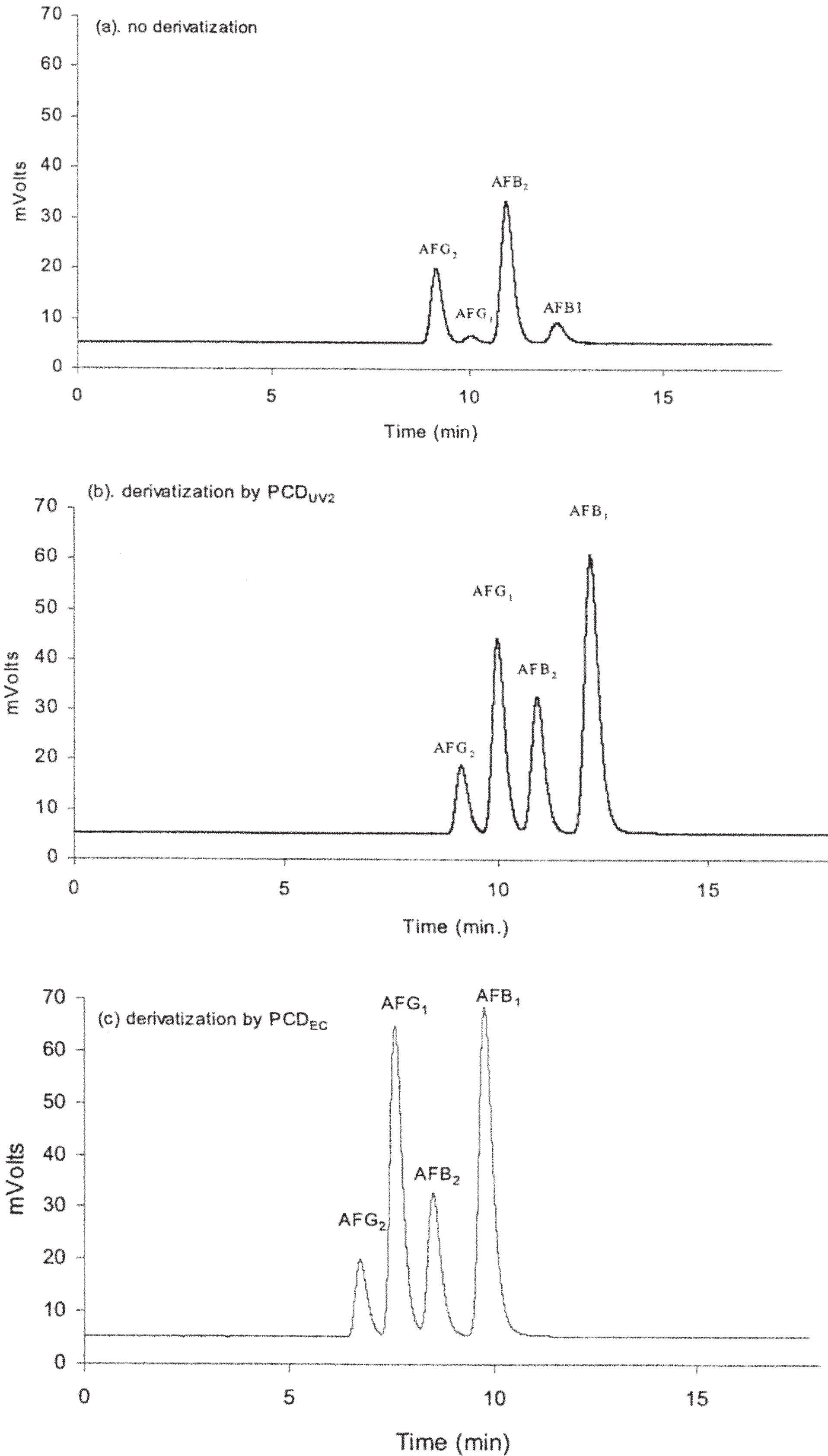

Figure 4. Chromatograms obtained for a mixed aflatoxin standard (AFB_1 and AFG_1, each at 20 ng/mL, and AFB_2 and AFG_2, each at 4 ng/mL) by using (a) no derivatization, (b) PCD_{UV2}, and (c) PCD_{EC}.

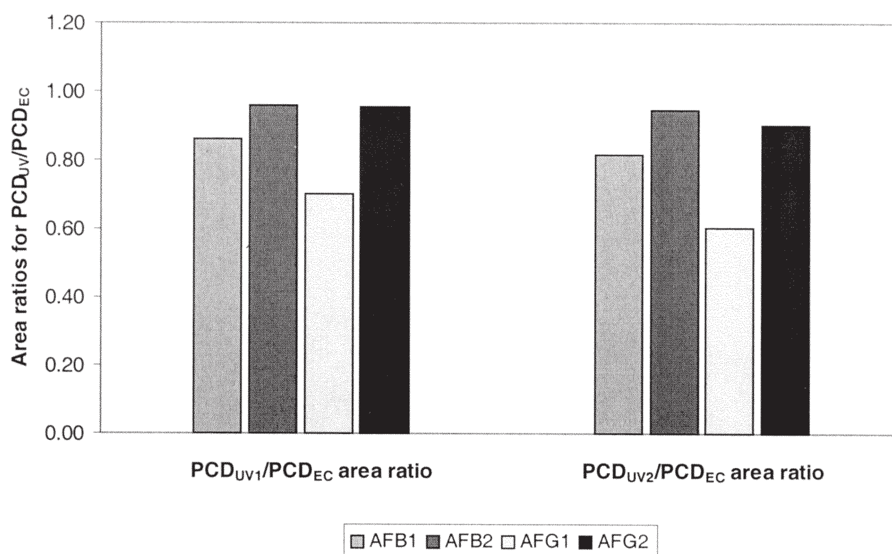

Figure 5. Graphic representation of the results obtained for a mixed aflatoxin standard for the PCD_{UV1}/PCD_{EC} and PCD_{UV2}/PCD_{EC} comparisons.

3. The valve was mounted in such a way that the power supply was disconnected when the Kobra cell was not in use.

Sample Extraction

Immunoaffinity cleanup was used for sample extraction according to the procedure described by Stroka et al. (24) for all food matrixes and according to the procedure described by Stroka et al. (25) for citrus pulp containing animal feed.

Standard Preparation

A mixed aflatoxin standard (AFB_1 and AFG_1, each at 100 ng/mL, and AFB_2 and AFG_2, each at 20 ng/mL) was used for spiking and for subsequent standard preparation. A mixed aflatoxin standard containing AFB_1 and AFG_1, each at 20 ng/mL, and AFB_2 and AFG_2, each at 4 ng/mL, was used systematically for testing the reproducibility of the results while both PCD systems were used.

Results and Discussion

We tested the various food matrixes for matrix interference when the 2 PCD techniques were used as described above. The fluorescence responses obtained by both PCD systems for AFB_1, AFB_2, AFG_1, and AFG_2 were compared directly, and the reproducibility of the results obtained by using either PCD technique (300 h for PCD_{UV1} and 343 h for PCD_{UV2}) was investigated by testing the course of the sensitivity of detection as a function of lamp use.

The enhancement of fluorescence by derivatization using both systems, for a mixed aflatoxin standard solution containing AFB_1 and AFG_1, each at 20 ng/mL, and AFB_2 and AFG_2, each at 4 ng/mL, is demonstrated in Figure 4. The difference in the retention times is due to the longer residence times of the substances in the reaction coils before they reach the fluorescence detector.

The first part of the comparison of the 2 PCD techniques was focused on differences in the possibility for matrix interference (interfering peaks, quenching effects). For all matrixes studied, a cleanup step using immunoaffinity columns was performed. This has been shown to be an effective cleanup mechanism for the determination of aflatoxins in various matrixes when bromination is used for PCD (24, 25). The results of the present work show that this conclusion is valid also for PCD by UV irradiation, even for matrixes such as hazelnuts, corn, and citrus pulp containing animal feed that was shown to cause matrix interferences in the past, even though

Table 1. Relative standard deviations (%) for each PCD device tested during the in-house validation study

PCD device tested	AFB_1	AFG_1	AFB_2	AFG_2
PCD_{UV1}	1.6	1.8	1.1	0.3
PCD_{UV2}	1.3	1	0.8	0.7
PCD_{EC}	2.0	0.9	1.2	1.1

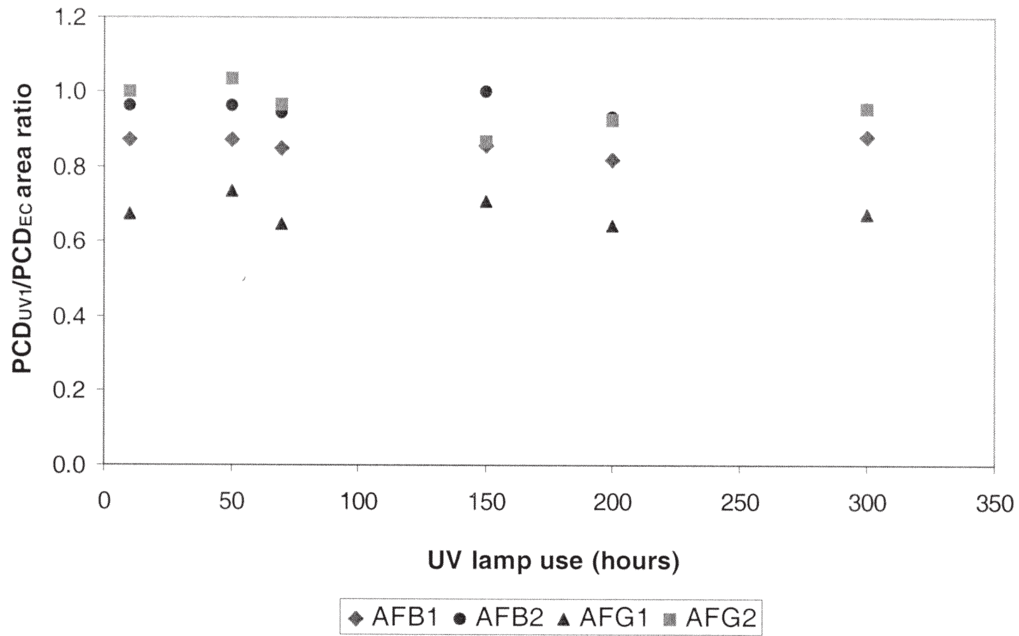

Figure 6. Graphic representation of the PCD_{UV1}/PCD_{EC} area ratio versus lamp use (h) for a mixed aflatoxin standard.

the extracts were not entirely purified by the immunoaffinity column cleanup. The 2 derivatization techniques were compared directly by determining the area ratios (PCD_{UV1}/PCD_{EC} and PCD_{UV2}/PCD_{EC}). The height response, which was not suitable for this type of comparison, was not used because it is dependent on void volume created by the setup.

The average ratios of PCD_{UV1}/PCD_{EC} and PCD_{UV2}/PCD_{EC} of all runs for standards, obtained by integration of area responses, are shown in Figure 5.

As shown from the ratios in Figure 5, AFB_1 and AFG_1 lead to lower values when the photochemical derivatization techniques are used than when the electrochemical bromination derivatization technique is used.

During the in-house validation study performed for the PCD_{UV} and the PCD_{EC} systems, it was demonstrated that the relative standard deviation (RSD) values obtained for multiple injections for each PCD_{UV} system are comparable to those obtained for the PCD_{EC} system. These values are presented in Table 1.

To investigate the efficiency of the UV lamps on a longer time scale, the lamps of PCD_{UV1} and PCD_{UV2} were tested for 300 and 343 h, respectively. The corresponding results (Fig-

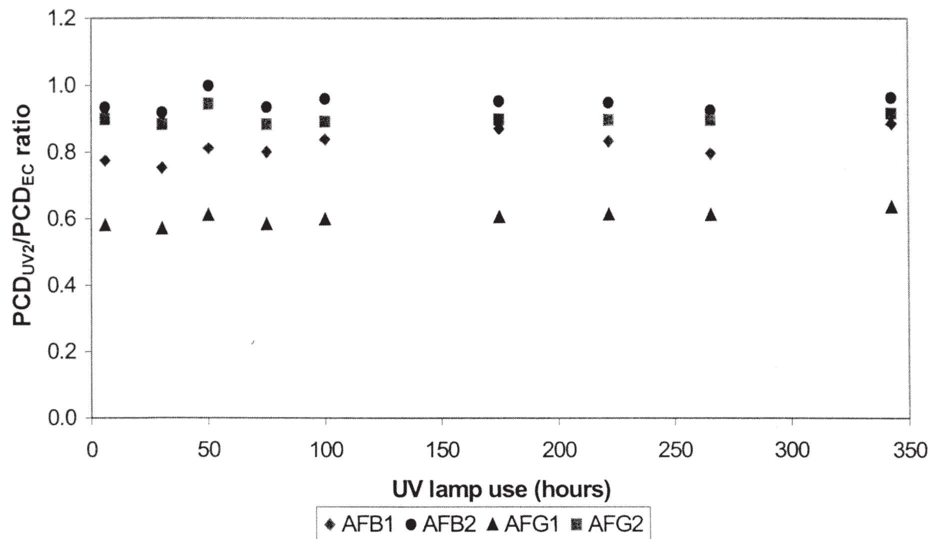

Figure 7. Graphic representation of the PCD_{UV2}/PCD_{EC} area ratio versus lamp use (h) for a mixed aflatoxin standard.

ures 6 and 7) show that the long-term use did not lead to decreased intensities of the fluorescence signals for either PCD_{UV} device. Each data point represents 5 replicates for the standard used (AFB_1 and AFG_1, each at 100 ng/mL, and AFB_2 and AFG_2, each at 20 ng/mL).

A decrease of 0.3 min in retention time for the peaks obtained by using PCD_{UV1} was observed during the first 20 h of use, and retention time remained stable thereafter. This decrease could be explained by a possible swelling of the PTFE material of the coil that created a smaller volume capacity. Nevertheless, the fluorescence intensities did not suffer any decrease and had no influence on the final results.

The PCD_{EC} device has been used for 3 years in our laboratory without any maintenance needed and without any changes in aflatoxin amplification. A limitation associated with this device is its sensitivity to 100% acetonitrile. As far as the PCD_{UV} devices are concerned, the manufacturers declare a lower UV lamp efficiency after 1000 working hours. At 1000 working hours, the efficiency is expected to be about 80% of the initial efficiency, depending on the temperature of use, with 25°C being the optimum temperature. Furthermore, the long-term use of the reaction coil (> 750 h) can also reduce the transmittance of UV light and therefore reduce the photochemical effects.

Conclusions

The comparison of the 2 PCD techniques demonstrates that both approaches, bromination and irradiation by UV light, are suitable for the determination of aflatoxins in various food and animal feed matrixes. Both UV systems tested showed comparable results for fluorescence amplification and repeatability, and their long-term use (300 h for PCD_{UV1} and 343 h for PCD_{UV2}) did not lead to decreased intensities of the signals.

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