Selection of a Suitable Method for Analysis of Aflatoxins in Date Fruits

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Fruits of the date palm (*Phoenix dactylifera* L.) are a vital component of the diet in most of the Arabian countries, but whether dates in the market are contaminated with aflatoxins is unknown. As screening will depend on accurate detection, the available AOAC Methods—with slight modification—for the recovery of aflatoxins were examined. The Romer minicolumn method failed to detect any aflatoxin in contaminated date fruits. Using the HPLC and postcolumn derivatization procedure, the contaminants branch (CB) method was found to give average recoveries of 75.7 and 83.5% for date varieties Lulu and Naghal, respectively. The recovery of total aflatoxins by the Best Food (BF) extraction and purification method was about 35% less than with the CB method.

Keywords: *Dates; aflatoxin; analysis*

INTRODUCTION

Date fruits are consumed in many forms and at all stages of the fruit development. In addition to the direct consumption of the fruit, dates are also utilized in many ways in modern industries (Mustafa et al., 1983; Sawaya et al., 1989; Shinwari, 1993).

The Arabian Peninsula is characterized by a long hot season, a relatively warm winter, and a very high humidity (more than 70%) prevailing throughout the year. These climatic conditions coupled with the physiochemical changes that take place in the date fruits during ripening make the date peculiarly liable to fungal attack. In practice, molds are considered to be the major causative agents of the spoilage of date fruits at all stages of ripening on trees as well as during storage and processing. Equally important are the facts that (a) *Aspergillus* was found to be the most abundant genus on dates at all stages of ripening (Al-Shaickly et al., 1986; Ahmed and Robinson, 1997); (b) in a preliminary, unpublished survey, which was carried out in our laboratory, aflatoxins were detected in one random sample of date fruits of Buchibal variety at Khalal stage at levels of 113 and 133 μg/g for B_1 and G_1, respectively; and (c) a previous study showed that dates can support the growth of toxigenic aspergilli and aflatoxin production (Ahmed et al., 1997).

The current position is that three AOAC methods, namely, the CB (Contaminants Branch), BF (Best Food), and RMC (Romer minicolumn) methods that employ chloroform, methanol, and acetone as extraction solvents, respectively, have become widely accepted. Collaborative studies provided the necessary measures of accuracy and precision for each method before it was adopted (Stoloff, 1977). However, although adoption of a method for specific commodity does not mean that it cannot be applied to other materials, it is necessary to evaluate the efficiency and suitability of the known methods when new products, such as dates, need to be examined. Consequently, it was decided to examine the suitability of the three modified AOAC methods for the detection of aflatoxins in dates, for only then could surveillance activities and the subsequent control of any aflatoxin problems in dates become feasible.

EXPERIMENTAL EVALUATION OF THE METHODS

(i) Materials. The variety Lulu, the most widely grown variety of date in the United Arab Emirates, was selected to study the suitability of the different methods available for the extraction of aflatoxins. The mature, full-colored and fleshy, Khalal stage, which proved to be the most susceptible to aflatoxin production (Ahmed et al., 1997), was chosen as substrate.

About 3−4 kg of the date fruits was surface sterilized for 1 min in an agitated vessel using 1% silver nitrate solution, which was then neutralized with 1% sodium chloride solution; subsequently, the fruits were rinsed three times with sterilized distilled water. After the pits were removed aseptically, the fruits were cut into small pieces and inoculated with a spore suspension (1×10^6 spores/mL) of *Aspergillus parasiticus* IMI 9109b—1 mL/100 g of fruit. The inoculated pieces were mixed well and distributed, as one layer, into sterile conical flasks of 1-L capacity (50 g each). The flasks were closed with cotton plugs and incubated at 28 °C for 10 days. During the first 3 days, the flasks were shaken twice daily to ensure uniform growth over the pieces. At the termination of the incubation period, all the flasks were autoclaved to de-activate the spores. The contents of the flasks were then transferred, carefully, to a large bowl (lower part of an empty desiccator) to which about 3 kg of uninoculated pieces was added. The contaminated material was then mixed with a hand-held, low-speed blender to give a uniform sample, which was distributed into screw-capped, disposable plastic bottles (about 100 g each) and stored at −18 °C for subsequent analysis.

At the time of analysis, and for each method, six bottles were removed from the freezer and allowed to reach room temperature before the required amount (50 g) was removed. The remaining fruit in each bottle was then decontaminated using 1.5% sodium hypochlorite according to the method described by Castegnaro et al. (1980).

BF Extraction and Cleanup Method. The BF method was carried out as described in AOAC Method 970.45 (1990) with some slight modifications that were done to suit the comparative nature of this study. The modifications included the following:

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(a) After extracting the sample with 250 mL of aqueous methanol (55/45, v/v), a 100-mL aliquot of the filtrate was taken to represent a 20-g sample (Stack, 1974).

(b) The 100 mL of filtrate was mixed with 50 mL of chloroform rather than the 25-mL aliquot described in the AOAC method.

(c) The chloroform phase was finally passed through Whatman No. 41 filter paper containing anhydrous sodium sulfate (4 g) to remove any water that might otherwise interfere with the final evaporation. The filter paper was then washed with fresh chloroform, and the extract was collected in a stainless steel beaker. The extract was evaporated in a boiling water bath to about 5 mL.

The concentrated extract was transferred quantitatively, with the aid of a Pasteur pipet, to a 4-dram vial, and the beaker was rinsed with a few milliliters of chloroform. The vial was placed over a water bath and the solvent evaporated to dryness under a gentle stream of nitrogen, prior to closure of the vial and storage in a freezer at −18 °C for TLC and HPLC determinations.

CB Extraction and Cleanup Method. The CB method (968.22) of the AOAC (1990) was subjected to the same modifications mentioned above, and the dried final extracts were kept in 4 dram vials and stored at −18 °C for TLC and HPLC determinations.

RMC Extraction and Cleanup Method. The AOAC (975.36) (1990) description of the RMC method was followed, but with a few minor modifications, namely:

(a) The minicolumn detection steps were omitted, as TLC and HPLC determinations were used throughout.

(b) The changes that were applied during evaluation of the BF method with respect to the amount of filtrate taken for analysis, the volume of chloroform used in the final transfer of the toxin from the extraction solvent, and the dehydration of the chloroform before the final evaporation step.

The resultant residue was transferred quantitatively to a 4-dram vial and stored in a freezer at −18 °C for TLC and HPLC determinations.

(ii) Analysis for Aflatoxin. Prior to the quantitative determination of the individual aflatoxins by high-pressure liquid chromatography, the aflatoxin content in the extract was estimated by thin layer chromatography as described in AOAC Method 968.22 (1990). The dry extracts of the samples were redissolved in the injection solvent (HPLC grade water: acetonitrile, 60/40 v/v), and the vials were protected from light and analyzed by HPLC on the same day.

The analysis was carried out using a Hewlett-Packard (HP) 1090 liquid chromatogram, equipped with PV5 Ternary Solvent Delivery System and three-channel pump. A stainless steel RP column (250 × 4.6 mm) packed with 5 μm Spherisorb ODS 1 (Aldrich Chemical Co., Gillingham, Dorset, England) was used. The mobile phase was water:acetonitrile:methanol (60/30/10 v/v/v, all HPLC grade) at a flow rate of 0.75 mL min^{-1}, and the analysis time was 21 min.

Postcolumn derivatization (PCD) with saturated iodine solution was carried out in a PCX 3100 reactor (Pickering Laboratories, Mountain View, CA) equipped with a stainless steel, zero dead volume T-fitting and 0.01 in. bore coil. Further details of the reaction conditions are given by Shepherd and Gilbert (1984).

The postcolumn reactor was connected to an HP-1046A programmable fluorescence detector (PFD); the excitation and emission wavelengths were 360 and 450 nm, respectively, with PMT gain of 12. The PFD response was automatically integrated in HP-9153 integrator and HP-79994A ChemStation using an Edit Calibration Table prepared by injecting external standards at the beginning of the run.

(iii) Percentage Recovery. To calculate, precisely, the percentage recovery of aflatoxins extracted by the CB method, two varieties of different chemical composition, namely, Naghal and Lulu (Ahmed et al., 1995), were chosen at the hard, raisin-like Tamr stage as suitable substrates. Tamr was chosen because it is the stage at which dates are mainly exported and handled in the International Trading System, and although dates at Tamr are not susceptible to infection, it can be assumed that aflatoxins produced at any earlier stage of the fruit development would remain stable into this final stage.

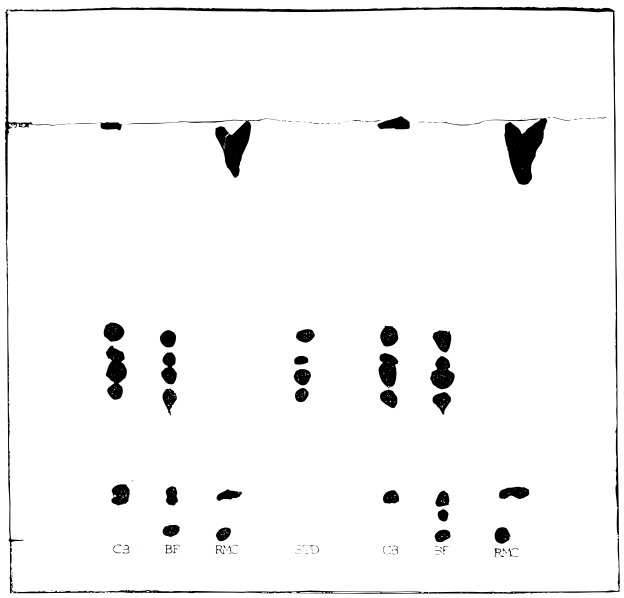

Figure 1. Photocopy of a TLC plate covered with transparent self-adhesive plastic film showing aflatoxins extracted from contaminated dates using the CB, BE, and RMC extraction and purification methods. A standard solution (STD) of aflatoxins was spotted centrally, and the four major aflatoxins were revealed, namely (from top to bottom), B_1, B_2, G_1, and G_2. The developing solvent was chloroform−acetone (9 v/v).

The selected fruits at the Tamr stage were cut into small pieces, and a sample of about 50 g was weighed into the container of a high-speed blender. Meanwhile, a standard solution of aflatoxins B_1, B_2, G_1, and G_2 was prepared as described in AOAC Method 971.22 (1990). Then a known amount of the standard solution was transferred into a 10-mL glass bottle and evaporated to dryness under a gentle stream of nitrogen. The dry residue was then redissolved in water:acetonitrile (9/1) to give a final concentration of 12.5 μg/mL for B_1 and G_1 and 2.5 μg/mL for B_2 and G_2. Exactly 1 mL of the prepared standard mixture was added dropwise to the sample in the blender jar using a 1000 μL micropipet (Brand, Germany). The jar was closed, and after mixing, the contents were kept in the dark for about 2 h to allow the aflatoxin to be absorbed by the date pieces; after which time, the extraction was carried out according to the CB method. Six replicates and an 'unspiked' control for each variety were extracted, and the average recoveries and standard deviations were calculated.

RESULTS AND DISCUSSION

To evaluate a method by which aflatoxins could be successfully extracted from date fruits, inoculation with toxigenic aspergilli was preferred to 'spiking' the material with a standard solution of aflatoxins. This choice was made because the growing mycelium of *Aspergillus* penetrates inside the date tissues and secretes both aflatoxins and other secondary metabolites; the latter, together with some substrate components, might have affected the extraction and purification steps used. It was concluded, therefore, that the analytical results would be more meaningful than those obtained with spiked samples (Trinder, 1985).

Surprisingly, as is clear from Figure 1, no aflatoxin was recovered from the contaminated date samples using the RMC extraction and cleanup method. Six replicates of the contaminated samples were used, and

neither the TLC nor the HPLC results showed any aflatoxin. The same procedure was repeated with another batch of six replicates, and again the results were negative.

The reason for these failures may be that the acetone failed to extract aflatoxin from the samples due to some intrinsic factor associated with date fruits or, most probably, it might be due to losses and/or degradation of aflatoxins that occurred during the purification procedure. Alternatively, it could be that the acetone extracted components that interfered with the separation of the aflatoxins, for it was observed that the acetone extract was more densely-colored than the extracts obtained by the other two methods. The color of the acetone extract remained darker than the original CB and BF extracts even after the ferric chloride purification. This observation indicated that the acetone was extracting more pigments and artifacts from the date samples than the methanol and chloroform used in the BF and CB methods, respectively. However, overcoming these problems associated with acetone as the extracting solvent was deemed to be beyond the scope of the study.

The same problem was reported by Swaminathan and Koehler (1976) when using acetone for the extraction of aflatoxins from potato, due to the formation of a colloidal suspension that was difficult to remove. Velasco (1970) concluded that adjustment of the pH in the gel formation step of the purification procedure was critical, for otherwise aflatoxins might be adsorbed onto the gel. He found also that losses of aflatoxin of up to 66% could occur due to changes in pH. These losses were attributed to either a loss of fluorescence or to aflatoxins becoming bound to other compounds in the natural extract, thus influencing their recovery. In addition, residues of the potassium hydroxide used to wash the chloroform could lead, during the drying process, to the decomposition of aflatoxins found in the final extract (Romer, 1975). It has been suggested also that acetone can cause decomposition of aflatoxins when added to the product and left for a period of time (Velasco and Morris, 1976).

Although the RMC method was declared to be applicable to many agricultural products (AOAC, 1990), it may not be suitable for certain crops, such as dates. Similarly, Romer (1975) failed to recover any aflatoxin with acetone from a sample of figs spiked with 15 μg/kg total aflatoxin.

With regard to the CB method, the most critical step, which can lead to 'false negatives', is the use of diethyl ether as first suggested by Pons and Goldblatt (1969); the ether tends to elute a portion of the aflatoxins from the column during the cleanup step. This situation was confirmed in our laboratory by collecting and drying the ether fraction, which was then redissolved in chloroform followed by TLC detection. However, the use of anhydrous ether overcomes this problem, as the difference between anhydrous and normal ether is in effect the difference between washing and elution; proper activation of the silica gel is also important to the process.

The BF method was found to be quicker than the CB, but emulsions sometimes occurred and caused problems in the separation of the layers; the addition of sodium chloride (3−5 g) helped to break these emulsions.

As shown in Figure 1, some fluorescing artifacts were seen on the TLC plates with both the CB and BF methods. These spots could be either natural constituents of the date fruits or other secondary metabolites secreted by the growing fungus. Luckily, these spots were located outside the aflatoxin region and, hence, created no problem with the identification of the four major aflatoxins.

Table 1. Comparison of the CB and BF Methods of Extracting Aflatoxins from Date Fruit Pieces of Lulu Variety at Khalal Stage Inoculated with *A. parasiticus* (IMI 91091b)[a]

method		AFB1	AFB2	AFG1	AFG2	total
BF	1	186	111	933	143	1373
	2	176	87	777	112	1151
	3	216	107	964	137	1424
	4	213	102	933	137	1386
	5	194	98	925	130	1347
	6	206	101	925	132	1364
	mean	198	101	910	132	1341
	SD	15	7	66	9	95
	CV, %	7.6	6.9	7.2	6.8	7.1
CB	1	451	141	1243	173	2008
	2	517	152	1464	183	2315
	3	454	133	1278	147	2011
	4	406	123	1150	149	1828
	5	432	127	1364	158	2080
	6	440	130	1324	163	2056
	mean	450	134	1304	162	2050
	SD	37	10	103	13	156
	CV, %	8.2	7.5	8	8	7.6

[a] All figures are in μg/kg.

Generally, as illustrated in Table 1, the total aflatoxin recovery by the BF extraction and purification method was about 35% less than with the CB method. This finding is in agreement with most of the reports comparing the CB and BF methods [e.g., Chang et al. (1979) on the same sample of peanut meal and Trinder (1985) on sorghum and peanuts]. Tested by many laboratories in a collaborative study, van Egmond and Wagstaffe (1989) reported that the BF method had the lowest extraction efficiency among the methods tested.

A further striking difference between the two methods was with respect to aflatoxin B_1 (Table 1), where the BF method yielded only about 44% of the amount of aflatoxin B_1 extracted by the CB method, followed by G_1 (70%), B_2 (75%), and G_2 (81%). This poor recovery of B_1 is in agreement with other reports (Shotwell et al., 1978; Chang et al., 1979).

It was noted also that both the CB and BF methods showed (Table 1) low coefficients of variations (CV) which, as a percentage of the mean, were in the range of 6.8−8; these low CVs indicate a high level of repeatability for both methods tested. The overall range of CVs is close to the values of 14% and 10.4% given by Boyacioglu and Gonul (1988) for the extraction of aflatoxins from raisins by the CB and BF methods, respectively. On the other hand, much higher CVs are given in the literature (Eppley et al., 1968; Waltking, 1970; Stack, 1974; Schuller et al., 1976), which reflect both within- and between-laboratory differences as well as the influences of the food products used.

The repeatability of the HPLC quantification stage was further tested by injecting two randomly selected samples of date extracts for 6 consecutive days; the samples were diluted to get the peaks within the 100% fluorescence range. As can be seen from Table 2, the HPLC results showed, as indicated by the low values of the CV %, good repeatability; generally, these results suggest that the quantification step contributes little to the variation in the results shown in Table 1.

Table 2. Results of Injecting Two Randomly Selected Samples of Contaminated Date Extract for 6 Consecutive Days To Test the Repeatability of the HPLC Determination Procedure[a]

		AFB1	AFB2	AFG1	AFG2	total
sample 1	1	19.1	0.71	10.8	ND	30.6
	2	24.6	0.99	13.7	ND	39.4
	3	23.9	0.95	13.5	ND	38.4
	4	24	0.91	13.5	ND	38.4
	5	25.8	1.04	12.6	ND	39.4
	6	24.7	1.0	11.8	ND	37.5
	mean	23.7	0.9	12.7	0.0	37.3
	SD	0.7	0.0	0.7	0.0	0.7
	CV, %	2.9	0.0	5.5	0.0	1.9
sample 2	1	29.9	1.39	19.5	0.41	50.9
	2	27.2	1.15	16.2	0.34	44.9
	3	26.9	1.09	15.8	0.32	44.1
	4	29.6	1.32	18.0	0.37	49.3
	5	29.1	1.38	18.1	0.37	49.0
	6	28.37	1.14	15.8	ND	45.3
	mean	28.5	1.3	17.2	0.4	47.3
	SD	1.0	0.1	1.0	0.0	2.2
	CV, %	3.5	7.6	5.8	0.0	4.7

[a] All figures are in μg/L.

Table 3. Recovery (%) of Aflatoxin from Spiked Samples of Date Fruits of Two Varieties at Tamer Stage[a]

variety		AFB1	AFB2	AFG1	AFG2	total mean recovery
Lulu	1	79.8	63.5	84.0	70.3	
	2	79.2	62.4	85.2	72.6	
	3	76.3	66.2	84.8	75.0	
	4	75.6	67.3	86.1	76.2	
	5	78.4	61.7	83.3	73.7	
	6	75.2	75.6	87.6	76.9	
	mean	77.4	66.1	85.2	74.1	
	SD	1.8	4.7	1.4	2.2	
						75.7
Naghal	1	85.6	84.1	87.8	85.4	
	2	83.8	75.6	86.1	84.2	
	3	80.5	82.7	89.3	80.7	
	4	86.2	81.7	88.3	78.6	
	5	89.3	79.1	85.5	76.0	
	6	82.2	73.7	91.8	85.9	
	mean	84.7	79.5	88.1	81.8	
	SD	2.8	3.8	2.1	3.7	
						83.5

[a] Extraction and purification was by the CB method.

The final phase of the evaluation was designed to determine the accuracy of the selected method (CB) through calculation of the mean recoveries from spiked samples of dates. The results in Table 3 indicate that the percentage recoveries can be considered satisfactory according to the guidelines of Schuller et al. (1976), who stated that any method that showed recovery of 70% or more could be eligible for future official adoption. However, it is worth noting the differences in total recovery between the two varieties, Lulu and Naghal, a contrast that could perhaps be attributed to differences in their chemical composition (Ahmed et al., 1995).

Given the good recovery of aflatoxin from the spiked samples, it can be concluded that, of the three methods tested, the CB method should be considered as the method of choice for the extraction of aflatoxins from dates and their determination by TLC or HPLC. However, further work on the BF method should not be ruled out, for it was found to be fast, easy, and gave a clean extract; its efficiency might be improved through repeated and extended extraction (Trinder, 1985). In addition, as dates contain a negligible amount of fat, the hexane cleanup step in both the CB and BF methods might be omitted to save time and cost.

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