ABSTRACT

A method has been developed for detection of aflatoxins, mycophenolic acid, patulin, penicillic acid, and sterigmatocystin in cheese. It is based on selective extraction with a mixture of equal volumes of 5% sodium chloride, methanol, and acetone, precipitation of caseins at −25°C, defatting with hexane, and removal of extraneous matter by transfer of mycotoxins to chloroform and ethyl acetate. The extract is purified further by column chromatography. Mycotoxins are quantitated on thin layer chromatograms by fluorescence comparisons. Mycophenolic acid, patulin, and penicillic acid are visualized with diethylamine. The limits of detection in cheese are about 20 μg/kg for mycophenolic acid, patulin, and sterigmatocystin, 30 μg/kg for penicillic acid, and 1 μg/kg for aflatoxins B₁ and M₁.

INTRODUCTION

Toxicological studies on the strains of fungi isolated from mold-ripened cheese led us to investigate the possible presence of mycophenolic acid and penicillic acid in marketed cheese. Results in our laboratory have shown that some varieties of cheese may contain significant mycophenolic acid (4). Olivigny and Bullerman (7) already speculated that certain strains of Penicillium roqueforti, which constitute the natural flora of cheese, can produce penicillic acid. In view of the biological properties and their classification as mycotoxins (3, 11), the presence of these two fungal metabolites in dairy products should be restricted as far as possible; a sensitive and reliable method for determination of these two microbial metabolites in cheese is required in the laboratory. A method has been devised by which mycophenolic acid and penicillic acid can be recovered quantitatively from cheese. Although the protocol described in this report was developed primarily for detection of the above two mycotoxins, it also may be used to detect aflatoxins, patulin, and sterigmatocystin.

EXPERIMENTAL PROCEDURE

Reagents and Materials

All solvents and reagents were of ACS grade. Thin layer chromatography was on Merck, Kieselgel 60 plates. Aflatoxins¹, patulin², penicillic acid², and sterigmatocystin¹ were obtained from commercial sources.

The standard of mycophenolic acid was prepared in the laboratory from a 15-day-old Penicillium brevi-compactum cultured on modified Czapek-Dox medium (1) at 25°C. Crude toxin was extracted from the mycelia and the culture medium with hot chloroform. The concentrated extract was chromatographed on a 20 × 450-mm column packed with 100 g of silicic acid (Mallinkrodt, 100 mesh). Mycophenolic acid was eluted with a gradient of chloroform:methanol (98:2, vol/vol to 70:30, vol/vol). The metabolite-containing fractions were collected and rechromatographed on a similar column by eluting with a gradient of n-hexane:acetone (90:10, vol/vol to 60:40, vol/vol). Mycophenolic acid-containing fractions were collected and purified by repeated crystallization. The purity and identity of the preparations were determined by thin layer chromatography, infra-red and mass spectrometry.
TABLE 1. Rf values of mycotoxins on silica gel plates.

<table>
<thead>
<tr>
<th>Mycotoxins</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aflatoxin B&lt;sub&gt;1&lt;/sub&gt;</td>
<td>.56</td>
<td>.75</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aflatoxin M&lt;sub&gt;1&lt;/sub&gt;</td>
<td>.09</td>
<td>.48</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mycophenolic acid</td>
<td>.39</td>
<td>.15</td>
<td></td>
<td></td>
<td>.22</td>
</tr>
<tr>
<td>Patulin</td>
<td>.32</td>
<td>.37</td>
<td></td>
<td></td>
<td>.22</td>
</tr>
<tr>
<td>Penicillic acid</td>
<td>.23</td>
<td>.22</td>
<td>.06</td>
<td></td>
<td>.90</td>
</tr>
<tr>
<td>Sterigmatocystin</td>
<td>.61</td>
<td>.53</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


Extraction of Toxins

A 25-g sample of cheese was blended with 100 ml of 5% NaCl solution for 2 min in a 1-liter Waring blender jar. The pH of the solution was adjusted to 6 by addition of one N acetic acid. Then 200 ml of methanol:acetone (50:50, vol/vol) were added, and the mixture was blended for additional 3 min. The mixture was filtered, and the filtrate was left at −20°C or at a temperature below −20°C for 5 to 6 h. The casein-precipitate formed by the action of cold was centrifuged at 2000 rpm for 20 min. Then 200 ml of the supernatant liquid were transferred to a 1-liter round-bottom flask and evaporated to about 75 ml in vacuo. The remaining solution was transferred quantitatively to a separatory funnel and washed with three successive 100-ml volumes of n-hexane. The defatted solution was extracted twice with 100 ml-volumes of chloroform: once with 100 ml of chloroform:ethyl acetate (50:50 vol/vol) and then with 100 ml of ethyl acetate, respectively. These extracts were combined and passed through a filter containing anhydrous sodium sulphate into a round-bottom flask and evaporated to about 5 ml. The combination was transferred to a small glass tube and evaporated to about 1 ml in a heating block under a stream of nitrogen. The silica gel was deactivated previously with 3% of water. The slurry was added to the chromatographic tube (22 × 400 mm) and firmly packed under pressure (500 mm of Hg). Then 100 ml of n-hexane:benzene (50:50 vol/vol) were added to the column to elute some of the impurities, and the additional extraneous matter was eliminated washing with another 100 ml of benzene.

Mycophenolic acid, patulin, penicillic acid, and sterigmatocystin were eluted with 300 ml of ether:n-hexane:formic acid (60:20:5, vol/vol). A subsequent 200-ml volume of benzene: ethanol (94.6, vol/vol) eluted the aflatoxins B<sub>1</sub> and M<sub>1</sub>. All elutions were under pressure. The two fractions were evaporated separately in a heating block under a stream of nitrogen and dissolved in 200 μl of chloroform.

Thin layer Chromatography

The TCL was in unlined, unequilibrated tanks. Quantitative analysis was by visual comparison with known standards. The developing solvent systems are indicated in the Table 1. Suitable chemical reagents were used for visualization of mycotoxin spots under ultraviolet light (Philips HPW 125 lamp).

Sterigmatocystin. Samples 5, 10 and 20 μl of the concentrated chloroform-solutions which contained sterigmatocystin were spotted on TLC plates along with the known standard solutions of sterigmatocystin. The plates were developed in solvent systems 1 and 2 (Table 1). A saturated solution of aluminium chloride in ethyl alcohol (20 g of AlCl<sub>3</sub> in 100 ml of ethanol) was sprayed on the chromatogram;
TABLE 2. Recovery of mycotoxins added to cheese.

<table>
<thead>
<tr>
<th>Mycotoxins</th>
<th>Added ( \mu g/kg )</th>
<th>Recovered (%) (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aflatoxin ( B_1 )</td>
<td>10</td>
<td>105 (10)</td>
</tr>
<tr>
<td>Aflatoxin ( M_1 )</td>
<td>10</td>
<td>100 (10)</td>
</tr>
<tr>
<td>Mycophenolic acid</td>
<td>50</td>
<td>116 (10)</td>
</tr>
<tr>
<td>Patulin</td>
<td>50</td>
<td>82 (20)</td>
</tr>
<tr>
<td>Penicillic acid</td>
<td>50</td>
<td>117 (10)</td>
</tr>
<tr>
<td>Sterigmatocystin</td>
<td>100</td>
<td>87 (20)</td>
</tr>
</tbody>
</table>

The later was heated at 80°C for 15 min. Sterigmatocystin, having Rf values as indicated in Table 1, appears as a bright yellow fluorescent spot under ultraviolet light. The concentration was measured by the fluorescence of the spots relative to that of the standard spots.

Mycophenolic Acid, Patulin, and Penicillic Acid. To approach the desired sensitivity with these three mycotoxins, two-dimensional TLC was used. The plates first were developed in system 3 (Table 1). In this solvent most of lipids and absorbing chromatographic background migrated while the three mycotoxins remained near the origin. The plates were developed in system 2 (Table 1). If interfering substances appeared at the level of mycotoxins, the second development could be carried out in either chloroform:acetone:water (93:7:1, vol/vol) or ether:hexane:90% formic acid (60:20:0.4, vol/vol). The plates were sprayed with diethylamine. Mycophenolic acid appeared as a fluorescent bright sky blue spot under longwave UV light. To reveal patulin and penicillic acid the plates were heated at 100°C for 15 min. Patulin appeared as a grey blue fluorescent spot and penicillic acid as a light blue fluorescent spot under longwave UV light. Once the mycotoxins were detected, they were quantified by visual comparison with known standards.

Aflatoxins \( B_1 \) and \( M_1 \)

This fraction was spotted along with the standard solutions of aflatoxins \( B_1 \) and \( M_1 \). The plates were developed in chloroform:acetone:water (93:7:1, vol/vol) for aflatoxin \( B_1 \) and Chloroform:acetone:propan-2-01 (85:10:5, vol/vol) for aflatoxin \( M_1 \). The toxins were estimated by visual matching with standards under longwave UV light.

RESULTS AND DISCUSSION

The method has been tested for its ability to estimate the six mycotoxins with different types of artificially contaminated cheese samples. The detection limits were 20 ppb for mycophenolic acid, patulin, and sterigmatocystin, 30 ppb for penicillic acid, and 1 ppb for aflatoxins \( B_1 \) and \( M_1 \). Thus, the detection limit for penicillic acid is much lower than those for other methods (2, 12). The amount for patulin is similar to that found by others (9) who worked with fruit juices. For aflatoxins and sterigmatocystin the limits are comparable to those by multimycotoxin detection methods (5, 10). There are no published methods for the estimation of mycophenolic acid in food and feed.

Diethylamine serves as a useful spray reagent in the analysis of mycophenolic acid, patulin, and penicillic acid. The percents in Table 2 show that the recovery of the fungal metabolites was as good as those with the other methods.

Many solvent systems in mycotoxin analysis do not separate mycophenolic acid, patulin, and penicillic acid from each other. They can have more or less similar Rf values. In this regard particular attention was paid to selection of the solvent balances in eluting systems. Furthermore, we noted under certain conditions that the specific spray reagent for patulin, 3-methylbenzo-2-thiazolone hydrazone hydrochloride (9), reacts similarly with penicillic acid giving yellow spots. Therefore, careful interpretation of the chromatograms was necessary to distinguish these three mycotoxins. The solvent systems 1 and 2 separated well and three toxins from each other.

Except for aflatoxins, no methods in the literature were designed specifically for the analysis of cheese for mycotoxins. Several extraction and clean-up procedures (8, 10) which had been used previously were investigated, but these did not give satisfactory recoveries. Acetone-water extraction and utilization of lead acetate to precipitate proteins did not extract mycophenolic acid and penicillic acid as they precipitated as their lead salts. In the present method, utilization of 5% NaCl-methanol-acetone as extracting solvents
and low temperatures help to precipitate most of the proteins. The hexane extraction and the subsequent partition of toxins to chloroform and ethyl acetate and the column chromatography eliminate further interfering substances.

The choice of the mycotoxins analyzed in this report was based on the potential in vitro capacity for the production of mycotoxins by strains of molds isolated from different types of cheese (6).

LITERATURE CITED