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EFFICIENCY OF CRUDE CORN EXTRACT CLEAN-UP ON DIFFERENT COLUMNS IN FUMONISINS DETERMINATION

ABSTRACT: The efficiencies of different clean-up procedures for crude corn extract from corn samples naturally contaminated by fumonisins B₁ and B₂ were compared. These procedures precede liquid chromatography determination with fluorescence detection. The efficiencies of immunoaffinity columns (IMA), strong anion exchange columns (SAX), as well as columns with reversed-phase C₁₈ (RP C₁₈) were investigated. No significant differences in the obtained results were found, regardless of the crude extract clean-up procedure. However, the use of IMA columns for clean-up provided better chromatographic resolution, with the clean-up procedure being the simplest and the fastest. Also, because of the possibility of IMA column regeneration, it is possible to prepare ten samples on one column, so all in all, the lower price of SAX and RP C₁₈ columns is of no great significance.

KEY WORDS: Clean-up extract (IMA, SAX, RP C₁₈ columns), fumonisins, liquid chromatography, maize

INTRODUCTION

Fumonisins are a group of compounds isolated for the first time from a fungus culture Fusarium moniliforme in 1988 (Gelderblom et al., 1988). They are produced by Fusarium moniliforme Sheldon (also known as Fusarium verticillioides (Sacc.) Nirenberg) and Fusarium proliferatum, which are widely spread in nature, and most frequently contaminate corn and corn products (WHO, 2000).

All quantitative techniques used for fumonisins determination, except enzyme-linked immunosorbent assay (ELISA) and in some cases liquid chromatography (LC) with mass detector, require clean-up of crude extract of the analyzed sample (Abramović et al., 2002; Jakšić, 2004). Cartridges with different sorbents are used for that purpose: columns filled with C₁₈ re-
verse phase (RP C_{18}), columns with strong anion exchange (SAX), as well as immunoaffinity (IMA) columns.

Comparison of efficiencies of the above said three types of columns for crude extract clean-up from different samples was the subject of several papers. For example, Müller and Gustavsson (2000) determined fumonisins B₁ (FB₁) and B₂ (FB₂) in different maize products by two different methods based on clean-up steps using an immunoaffinity column and a combination of SAX and C_{18} columns, respectively. Recovery, repeatability, and results from the survey showed comparable results among these methods. Furthermore, De Girolamo et al. (2001) compared the efficiency of clean-up of extracts from different maize products on SAX and IMA columns. They found that the use of SAX clean-up column for maize flour, muffins and infant formula gave better fumonisins recoveries than IMA clean-up, while this was not the case with corn flakes and extruded maize. However, a peak interfering with FB₁ appears in the chromatograms of some extracts when a SAX column is used for clean-up, which, according to the authors makes the use of IMA columns obligatory.

Continuing our previously begun research (Abramović et al., 2005), the aim of this work was to compare the efficiencies of all three types of columns in clean-up of spiked corn extract, as well as to investigate the possibility of multiple use of SAX and RP C_{18} columns, i.e. their regeneration. Finally, the investigations were performed using crude extract of corn samples naturally contaminated with fumonisins B₁ and B₂ in respect to recovery of fumonisins and chromatography resolution.

**MATERIAL AND METHODS**

All solvents used for fumonisins extraction from corn samples, as well as those for the mobile phase preparation were of LC grade. All chemicals used in the investigation were of reagent grade. Solutions were prepared in deionized water except when stated otherwise.

*Fumonisins calibrant solutions.* Fumonisins B₁ (Sigma, from Fusarium moniliforme, approx. 98% TLC) and B₂ (Sigma, from Fusarium moniliforme) were purchased as analytical standards. Calibrant solutions were prepared in acetonitrile-water (50:50, v/v) at concentration of 100 μg/ml for FB₁ and FB₂. Fumonisins calibrant solutions are stable up to 6 months when stored at 4°C (Visconti et al., 2001). Stock solution containing FB₁ at 10 ng/μl and FB₂ at 5 ng/μl was prepared by measuring 500 μl calibrant solution FB₁ and 250 μl calibrant solution FB₂ into 5 ml volumetric flasks and diluting to volume with acetonitrile-water (50:50, v/v). Working calibrant solutions were prepared by appropriate dilution of stock solutions with acetonitrile-water (50:50, v/v). Standard solutions were stored at 4°C.

*Preparation of phosphate-buffered saline (PBS).* 8.0 g NaCl, 1.2 g anhydrous Na₂HPO₄, 0.2 g KH₂PO₄, and 0.2 g KCl were dissolved in about 990 ml water, pH was adjusted to 7.0 and the solution was diluted to 1 l.
Preparation of o-phthalaldehyde-2-mercaptoethanol reagent. 40 mg o-phthalaldehyde (OPA, Sigma, min. 99%) were dissolved in 1 ml methanol, diluted with 5 ml 0.1 mol/l Na₂B₄O₇, and 50 ml 2-mercaptoethanol (MCE, Serva) were added. This reagent is stable up to 8 days in a capped, aluminum foil-covered vial in the dark.

LC mobile phase. Methanol-0.1 mol/l NaH₂PO₄ (Merck, extra pure) (78:22, v/v), with pH adjustment to 3.3 with o-phosphoric acid. The mobile phase was filtered through a 0.45 mm membrane (ISO-DISC™ Filters PTFE 25-4, Supelco).

Sample preparation. Corn samples were collected during autumn of 2002 from four localities in Vojvodina. Immediately after sampling, 1000 g of each sample were prepared by grinding in a laboratory mill in such a way that 93% passes through a sieve with pore diameter of 0.8 mm. After that, the sample was homogenized by mixing. Samples prepared in such a way were packed in plastic bags and stored in a freezer at −20°C until analysis. Prior to each analysis, the samples were allowed to reach room temperature.

Principle of determination

Fumonisins were extracted from corn with acetonitrile-methanol-water. After filtration (and dilution), the crude extract was cleaned-up on one of the columns. The final sample extract was derivatized with o-phthalaldehyde and 2-mercaptoethanol and analyzed by reversed-phase liquid chromatography with fluorescence detector.

Extraction. 20.0 g samples were extracted twice with 50 ml of acetonitrile-methanol-water (25:25:50, v/v/v) in a laboratory blender for two minutes. The combined extracts were centrifuged at 3000 x g for 10 min, with subsequent supernatant filtration through filter paper (Macherey-Nagel, Type 751, Düren, Germany).

Clean-up of raw extract

Immunoaffinity column clean-up. 10 ml of the filtered extract was diluted with 40 ml of PBS. The diluted extract was then filtered through a 1.0-µm microfiber filter (Vicam, Watertown, MA, USA). 10 ml of the filtrate were then applied to an IMA column (FumoniTest™, Vicam, Watertown, MA, USA). After rinsing of the column with 10 ml of PBS, fumonisins were eluted using 3 ml of methanol (MeOH), at a rate of 1 drop per 10—15 seconds. The eluate was evaporated just to dryness at 60°C. Purified residue was redissolved in 200 µl acetonitrile-water (50:50, v/v).

Regeneration of the IMA columns. One IMA column was used five times in a row. Each time, after the elution of toxins, the diluted extract was applied to the same column. After that, the column was washed with 10 ml of PBS solution, leaving a part of the solution on the column. The column was regenerated at 4°C for 24 h.
**SAX column clean-up.** 2.5 ml of the filtered extract were applied to a SAX column (LC-SAX SPE Supelclean™, Supelco, Bellefonte, USA, capacity 3 ml, with a 500 mg filling) which was previously conditioned with 3 ml MeOH and 3 ml of mixture MeOH-water (3:1, v/v). The column was rinsed with 3 ml of mixture MeOH-water (3:1, v/v) and 3 ml MeOH. After that, fumonisins were eluted from the column with 3 ml of mixture MeOH-acetic acid (99:1, v/v). The rate of elution was 1 drop per 10—15 seconds. The eluate was evaporated just to dryness at 80°C, 1—2 ml of MeOH were added and once again evaporated to dryness. Purified residue was redissolved in 200 µl acetonitrile-water (50:50, v/v).

**Regeneration of the SAX columns.** One SAX column was used five times in a row. Each time, after the elution of toxins, the raw extract was applied to the same column. After that, the column was regenerated by washing with 5 ml of 0.1 mol/l water solution of HCl and 8 ml water, filled with MeOH and left at room temperature for 24 h.

**RP C$_{18}$ column clean-up.** 5 ml of the filtered extract were diluted with 12 ml of 1% KCl solution and filtered through 1.0-µm microfiber filter paper. After that, 8.5 ml of the diluted extract were applied to a RP C$_{18}$ column (LC-18 SPE Tubes Supelclean™, Supelco, Bellefonte, USA, ~10% C, endcapped, capacity 6 ml, with a 1 g filling), previously conditioned with 5 ml MeOH and 5 ml 1% KCl solution. The column was eluted with 10 ml of acetonitrile-1% KCl (2:8, v/v) mixture. Fumonisins were subsequently eluted with 12 ml of mixture acetonitrile-water (7:3, v/v), the rate of elution being 1 drop per 10—15 seconds. The eluate was evaporated just to dryness at 60°C. Purified residue was redissolved in 200 µl acetonitrile-water (50:50, v/v).

**Derivatization and liquid chromatography**

A 50 µl aliquot of the extract was mixed with 50 µl of the OPA-MCE reagent at room temperature with the reaction time of one minute with stirring. 20 µl of derivatized solution were injected into the LC system. The equipment consisted of an LC system — BioRad 2800 with Supelcosil™ LC-18-DB column (250 x 4.6 mm id, particle size 5 mm) with a fluorescence detector Hewlett Packard 1046A, response time 4 s, flash frequency 220 Hz. LC pump delivered 1 ml/min constant flow rate. Wavelength of excitation radiation was 220 nm and emission 440 nm.

**RESULTS AND DISCUSSION**

In our previous work, optimal conditions were established for separation and determination of fumonisins by the LC method, as well as the clean-up efficiency of IMA columns for spiked corn sample crude extract (Abramović et al., 2005). The possibility of multiple use and regeneration of IMA columns was investigated as well. It was found that the efficiency of successive clean-up on one IMA column for FB$_1$ is 89.0±7.1%, and for FB$_2$ 88.5±1.3%,

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i.e. after column regeneration 88.5±11.6% for FB₁ and 92.5±7.5% for FB₂ (the average of 5 measurements in both cases).

The study of efficiency of crude spiked corn sample extract clean-up on SAX and RP C₁₈ columns, as well as the possibility of their multiple use and regeneration was continued in this work. It was found that SAX columns can be used five times in a row and at least once more after regeneration. The achieved efficiency of SAX columns is higher and has a value of 109.4±6.9% for FB₁ and 92.1±6.8% for FB₂ (the average of 5 measurements), and after regeneration 109.7% and 95.9%. However, the clean-up procedure is significantly more complicated and long-lasting in comparison to IMA columns.

When the possibility of multiple use and regeneration of RP C₁₈ columns was investigated, it was found that they can be used only once, as well as that they can’t be regenerated. Also, the efficiency of FB₁ and FB₂ determination in the spiked corn sample after crude extract clean-up is the lowest on RP C₁₈ columns, its value being 82.4±9.2% for FB₁ and 74.5±5.2% for FB₂.

At the end, all three clean-up procedures for crude extract of corn samples naturally contaminated with FB₁ and FB₂ used for LC determination were compared in this work. Chromatograms for corn sample with the highest fumonisins content after crude extract clean-up by use of all three types of columns are presented in Fig. 1, and the results of fumonisins determination are given in Table 1. As can be seen, chromatographic separation of FB₁ peak from the matrix peaks is poorer in the case of eluate from SAX and RP C₁₈ columns.

Tab. 1 — The influence of crude extract clean-up procedure on the results of fumonisins determination in corn

<table>
<thead>
<tr>
<th>Locality</th>
<th>Crop</th>
<th>Clean-up procedure</th>
<th>FB₁ content (mg/g)</th>
<th>FB₂ content (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kikinda</td>
<td>2002</td>
<td>IMA</td>
<td>&lt; 0.02</td>
<td>&lt; 0.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SAX</td>
<td>&lt; 0.02</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RP C₁₈</td>
<td>0.06</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Bačka Topola</td>
<td>2001</td>
<td>IMA</td>
<td>0.13</td>
<td>&lt; 0.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SAX</td>
<td>0.12</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RP C₁₈</td>
<td>0.07</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Subotica</td>
<td>2002</td>
<td>IMA</td>
<td>0.33</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SAX</td>
<td>0.34</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RP C₁₈</td>
<td>0.30</td>
<td>0.09</td>
</tr>
<tr>
<td>Bač</td>
<td>2002</td>
<td>IMA</td>
<td>0.79</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SAX</td>
<td>0.61</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RP C₁₈</td>
<td>0.70</td>
<td>0.18</td>
</tr>
</tbody>
</table>

In chromatograms presented in Fig. 1 a peak appearing immediately before FB₂ can be noted. From the results of authors who also determined FB₃ (Sydenham et al., 1992; Fazekas et al., 2000; VICAM, 1997), because of the similarity in the appearance of chromatograms, its presence in the sample analyzed in this work can be presumed. Unfortunately, due to the lack of the standard we weren’t able to confirm this presumption.
Fig. 1. Chromatograms of naturally contaminated corn sample (Bać 2002) obtained by application of different extract clean-up procedures: (A) IMA column; (B) SAX column and (C) RP C18 column.
As can be seen in Table 1, there is no significant difference in the obtained results for fumonisins content, regardless of the crude extract clean-up procedure.

From the above results, it can be concluded that the use of IMA columns for clean-up of crude extract of naturally contaminated corn samples and LC with fluorescence detection provide the best results. Aside from the fact that the purest extract was obtained by clean-up of crude corn extract on IMA columns, the clean-up procedure is the simplest and the fastest. Also, because of the possibility of regeneration of IMA columns (Abramović et al., 2005), it is possible to prepare more samples using one column. Therefore, having all said in mind, lower price of SAX and RP C_{18} columns is not of great significance.

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ЕФИКАСНОСТ ПРЕЧИŠЋАВАЊА СИРОВОГ ЕКСТРАКТА КУКУРУЗА ПРИМЕНОМ РАЗЛИЧИЋИХ КОЛОНА ПРИ ОДРЕЂИВАЊУ ФУМОНИЗИНА

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Резиме

Фумонизини су група једнине сврстане у фузарийјумске микотоксине. Чести су контаминенти кукуруза и производа од кукуруза. Поред тога што изазивају токсичне ефekte код различитих врста животиња, сматра се да изазивају канцер једњакакод људи. У литератури су описане различите технике за одређивање фумонизина, као и различите методе одређивања применом исте технике. С обзиром на чињеницу да су фумонизини присутни у веома малим концентрацијама а у веома комплексним медијумима, један од најзначајнијих корака у анализи је припрема, односно пречишћавање сировог екстракта узорка.

Примењујући претходно утврђене оптималне услове за одређивање фумонизина B₁ и B₂ течном хроматографијом са флуроресцентним детектором, у овом раду је упоређена ефикасност различитих начина пречишћавања сировог екстракта из узорка кукуруза које претходно њиховом одређивању. Испитана је ефикасност одређивања фумонизина у природно контаминацијним узорцима кукуруза након пречишћавања сировог екстракта на имуноафинитетним (IMA) колонама, јаким анјонским изменљивачким (SAX) колонама, као и на колонама са C₁₈ реверзном фазом (RP C₁₈).

На основу постигнутих резултата испитивања може се констатовати да нема сигнфикантне разлике у добијеним резултатима садржаја фумонизина независно од начина пречишћавања сировог екстракта. Међутим, примена IMA колона за пречишћавање даје најчистији екстракт, а сам поступак пречишћавања је најједноставнији и најбржи. Такође, због могућности регенерације IMA колона, могуће је припремити десет узорака на једној колони, те узимајући све наведено у обзир, нижа цена SAX и RP C₁₈ колона нема већег значаја.