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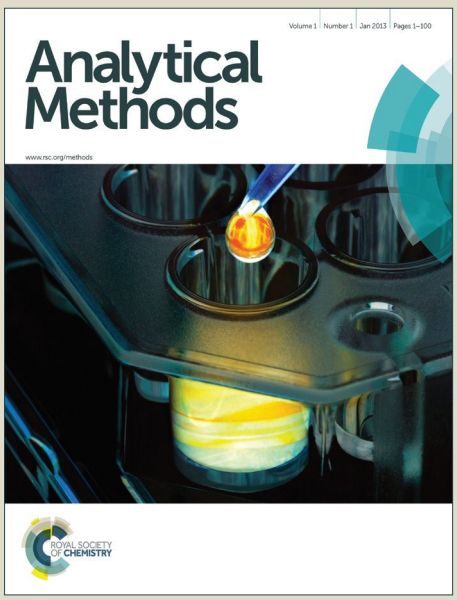

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Magnetic nanoparticles solid phase extraction- HPLC-UV for determination of deoxynivalenol from wheat flour

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Deoxynivalenol has been associated with human gastroenteritis and its presence in foods can cause clinical or subclinical manifestations to humans and animals. In this study, a fast, simple and validated extraction method of DON using Fe_3O_4 magnetic nanoparticles (MNPs) was investigated and compared with immunoaffinity column cleanup (IAC) process. The clean-up and extraction process was merged in a single step by mixing MNPs, extraction solvents and wheat extract. Possible impact parameters such as the amount of magnetic adsorbent, type and volume of extraction solvents, extraction time, salt addition and sample pH were investigated and optimized. Under the optimum conditions, DON was extracted and analyzed using high performance liquid chromatography with ultravoilet detection. The limit of detection (LOD) and quantification (LOQ) were 45 and 150 µg/kg, respectively. The proposed method was applied to real wheat flour samples and also satisfactory recoveries were achieved by analyzing the spiked sample at concentration levels of 500, 1000, 1500 µg/kg ranging from 78.3±5.4% to 93.8 ± 7.5%. The obtained results indicate that proposed procedure could be used as an easy, rapid and economic method for extraction of DON in wheat samples.

Introduction

There is a rising worldwide awareness of the serious cost that undesirable levels of mycotoxins may have on humans and animals, the presence of mycotoxins in food and feed is a common problem in all over the world. Deoxynivalenol (DON) is one of the type B trichothecene mycotoxins produced by Fusarium species and considered being the most commonly occurring hazardous mycotoxin in food and feed-stuff. Based on recent worldwide survey data, more than 50% of wheat, maize and oats analyzed are contaminated by this toxin [1, 2]. Among the trichothecenes, DON is detected most frequently worldwide and at highest concentrations in cereal grains from Poland, Germany, Japan, Iran, New Zealand, and the Americas[3]. DON has been associated with human gastroenteritis and its presence in foods can cause clinical or subclinical manifestations to humans and livestock [4, 5]. The European Commission (EC) established regulatory limits for this mycotoxin in cereal grains and cereal-based products [6, 7]. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) proposed a provisional maximum tolerable daily intake for DON of 1 µg/kg b.w. per day [8]. Around 600 million tons of wheat is produced in the world each year and most of them are converted to wheat flour for human consumption.

Sample pre-treatment are often necessary prior to analytical procedures and are also the main source of error of the analytical method. In past decades several sample preparation methods such as solid phase extraction (SPE) columns particularly C_{18} and immunoaffinity columns (IACs) followed by high-performance liquid chromatography (HPLC) with ultraviolet (UV) or mass spectrometry (MS) detectors, have been developed for cleanup and extraction of DON from food and feed [9-11]. However, most SPE adsorbents retain analytes by non-specific, hydrophobic interactions. This might lead to co-extraction of analytes as well as matrix interferences. Also, IACs have been prepared for mycotoxin determination but these columns are so expensive, disposable and have low stability against organic solvents and pH. Traditional pre-treatment methods have some disadvantages such as require large amounts of toxic organic solvents are relatively expensive, tedious and time consuming. Recently some novel, fast and inexpensive methods such as Dispersive Liquid-Liquid Micro Extraction (DLLME) have been developed for analysis of mycotoxins, that use low volumes of disperser and extractant solvents for extraction of mycotoxins [12, 13]. To date nano-sized materials have attracted increasing consideration in the scientific community. Nanoparticles (NPs) have a wide range of potential applications because of their large surface area and high active surface sites[14-16]. Magnetic nanoparticles (MNPs), a new kind of NPs material are super paramagnetic, which can easily be separated out of sample solution using an external magnetic field and retain no residual magnetism after the field is removed [17-20]. In the present research, we use Fe_3O_4 MNPs as cleanup materials along with mixed microliter amounts of chloroform and acetonitrile as extractant for extraction of

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deoxynivalenol from wheat flour. Simultaneously purification and extraction made sample pre-treatment simple and quick.

Materials and Methods

Reagents and Standard

HPLC grade water, acetonitrile, methanol, and all analytical grade extraction solvents were purchased from Merck (Germany). Iron oxide (II, III) magnetic nanoparticles was purchased from Sigma-Aldrich (Germany). Immunoaffinity columns, DONPREP®, and certified reference materials (CRM) for DON in wheat flour (2,000±400 µg/kg) were obtained from R-Biopharm Rhone LTD. The one milligram DON standard was obtained from Sigma Aldrich (St. Louis, MO, USA), the stock standard solution of DON (~25 µg/ml) was prepared in acetonitrile, then quantified by measuring its respective absorbance at 217 nm by UV spectrometry. This standard was stored in amber glass vial at −20 °C [1]. By dilution of appropriate amounts of this standard solution in the final MNPs extraction solution of blank wheat flour sample, the calibration curve was achieved.

Instrumentation and chromatographic conditions

A high-performance liquid chromatography system equipped with auto sampler (Waters 717), binary HPLC pump (Waters 1525), and a dual λ absorbance UV detector (Waters 2487) was used for the analysis. A reverse-phase column (Waters Nova-pak® C-18, 3.9×150 mm, 4 µm particle size, Waters, Milford, MA, USA) was used for separation at 40 °C. A mixture of water/methanol (90/10 v/v) at a flow rate of 1.1 mL min^{-1} was used as mobile phase in isocratic elution. The detection was performed at the wavelength of 217 nm [1].

Sample Preparation

Five kilograms of wheat sample was finely ground, in a Romer grinding/sub-sampling mill (Romer Series IITM mill; Romer Labs, inc. Union, MO, USA). The ground sample was passed through a 20-mesh sieve; a 100 g subsample was taken from each main (ground) sample and stored at -20°C. Twenty-gram wheat flour was blended with 100 mL distilled water at high speed (21,000 rpm, 3min) (Waring 4-Liter Laboratory Blender-USA), then the supernatant was filtered through Whatman no. 1 filter paper and 2 ml of the clear liquid filtrate was used for the extraction procedure.

Immunoaffinity column extraction

Two millilitres of the extract samples were passed through DONPREP® immunoaffinity columns with flow rate of one drop per second. The columns were washed with 5 mL of phosphate buffer solution with pH 7.4 at a same flow rate. Then, DON was eluted with 1.5 mL of methanol, then methanol was evaporated at 40 °C, and the residue was reconstituted in 1 mL mobile phase. Finally, 100 µL of the samples were injected into the HPLC system [13].

MNPs Extraction procedure

The extraction procedure was carried out as follows; two millilitre of the clear aliquot was placed in a 10-mL test tube. Then 0.05 g MNPs, 800 µL acetonitrile and 250 µL chloroform were added into the sample solution. The mixture was shaken for 6 min and then centrifuged for 3 min at 3500 rpm. Subsequently, the MNPs were isolated by placing a strong magnet and poured away. Afterward, deposited enriched organic solvents were withdrawn by syringe and evaporated and reconstituted in 1 mL HPLC grade water/methanol (90/10 v/v). Finally, aliquot of 100 µL was injected into the HPLC system.

Analysis of real samples

To evaluate the efficiency of the procedure, the optimized method was applied for the quantitative determination of DON in 5 wheat samples obtained from retail store in May 2015 (Mazandaran, Iran) and IAC method was used for comparison of the new method results [3].

Method validation

The method was validated for linearity, limit of detection and limit of quantification, precision by using CRM, and recoveries of spiked sample at 3 levels in inter-day and between days.

Results and Discussion

In preliminary studies, a series of spiked samples were prepared at a concentration level of 1000 ng/mL (3 times repeat to check any variable). Statistical analysis of results was done to evaluate statistically significant differences for data obtained for each factor using Student's t-test (SPSS version 17.0 Inc., Chicago, IL, USA) at 95% confidence level. The best response for each factor was used in subsequent experiments.

Effect of extraction solvent

The effect of extraction solvents on efficiency was studied using 800 µL of different organic solvents such as acetonitrile, ethanol, acetone and 250 µL chloroform as an auxiliary solvent and 0.05 g Fe_3O_4 NPs. Acetonitrile, ethanol and acetone were used as extractant but these extraction solvents are miscible with organic and aqueous phases and are not isolated from aqueous solution when used alone. For this reason, we used a mixture of two solvents, one of the solvents (acetonitrile, ethanol or acetone) used for extraction of DON and chloroform (the second solvent) served as an auxiliary solvent to isolate the extraction solvent from aqueous phase (Fig. 1a). Then the volume of acetonitrile was optimized in the range of 250-1000 µL by using 250 µL chloroform and 0.05 g MNPs. Based on the obtained results (Fig. 1b) the best recovery was achieved by 800 µL acetonitrile. Reduction in the extraction efficiency when the volume of acetonitrile exceeded 800 µL was attributed to increase the solubility of acetonitrile in aqueous phase. In the next stage, the volume of chloroform was studied in the range of 100-500 µL using a series of standard solutions extracted with 800 µL acetonitrile and 0.05 g MNPs. The best recovery with minimal consumption of chloroform was achieved by 250 µL chloroform (Fig. 1c).

Effect of MNPs

The effect of MNPs on the extraction efficiency was studied by varying the amount of Fe_3O_4 NPs in the range of 0-0.1 g. DON

has limited interest to adsorb on the surface of Fe_3O_4 NPs. Reducing the matrix effects in wheat extract can help to increase the extraction recovery of DON into organic extractant. In this study, we used bare Fe_3O_4 NPs to remove impurities in the wheat extract while the analyte extraction was done using a mixture of organic solvents. Without using MNPs, unclear deposited droplets of extraction solvents and recovery values lower than those obtained when MNPs were used (reduction of about 12%) were observed (Fig. 2a). The experimental results obviously showed that adding MNPs was necessary for effective extraction. Also, further increasing from 0.05 g MNPs did not have significant effect on extraction efficiency (Fig. 2b). So, 0.05 g Fe_3O_4 NPs was used for subsequent experiments.

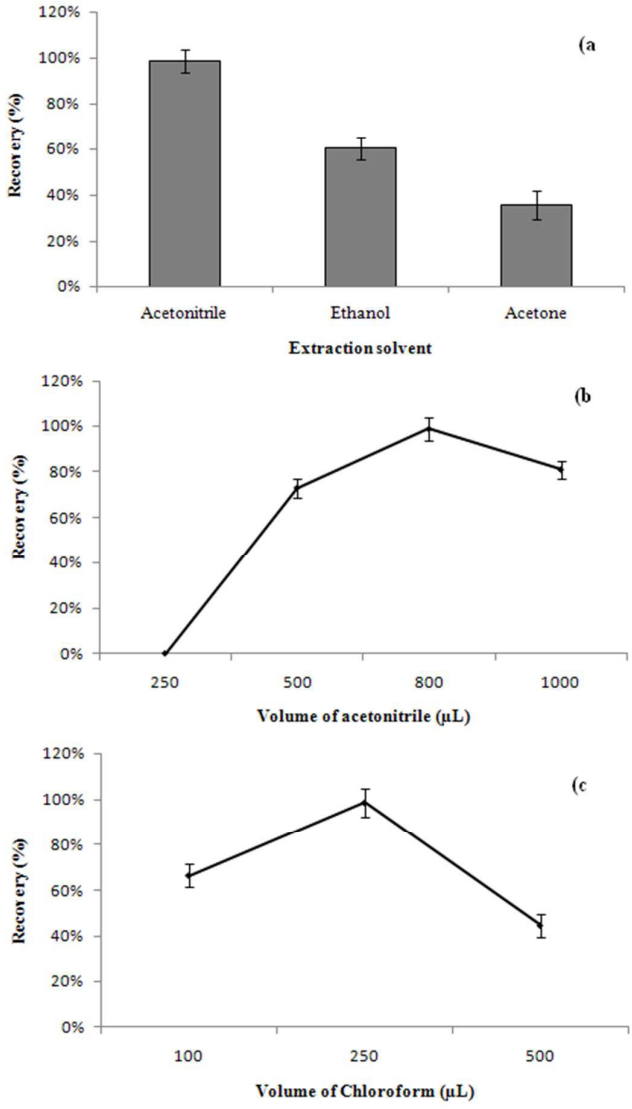

Fig. 1. a) Effect of extraction solvent on extraction efficiency b) Effect of volume of acetonitrile on extraction efficiency c) Effect of chloroform on extraction efficiency

Effect of extraction time

The effect of the extraction time on the extraction recovery of DON was optimized in the range of 1-10 min. Due to the large contact area between extraction solvent and analyte, and short diffusion route for NPs to adsorb impurities, the extraction was achieved in little time. The obtained results showed that extraction time more than 6 min did not have significant effect on the extraction recovery (Fig. 3).

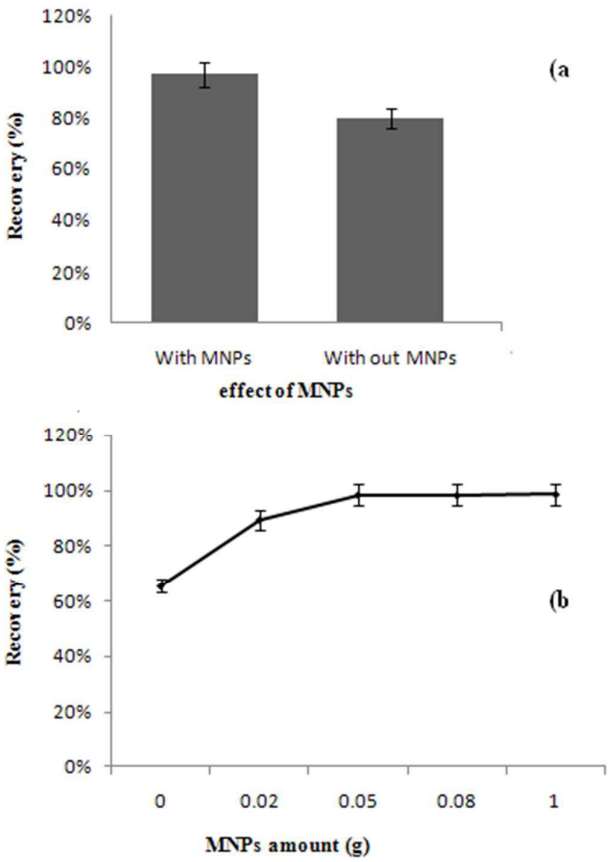

Fig. 2. a) Comparison of extraction efficiency with and without using MNPs b) Effect of MNPs on extraction efficiency (n=3) b) Effect of MNPs amount on extraction efficiency (n=3)

Effect of Sample pH

The pH value of the solution plays a critical role especially in case of ionisable compounds that may affect the extraction efficiency. The pH effect was studied in the range of 3.0-9.0 by adding diluted hydrochloric acid and sodium hydroxide solution, (Fig. 4). The best performance was obtained in the natural pH of wheat extract (about 7). Since at very low pH, the magnetic Fe3O4 nanoparticles can be dissolved or oxidized to maghemite phase; in other case at high pH, DON may hydrolysis and degradation may occur. So, further study was done at pH 7.0.

Effect of salt addition

In general, the solubility of analytes in aqueous solutions may be affected by ionic strength of the media due to salting out effect. The influence of salt addition on the extraction recovery of DON was studied by adding NaCl (0–10%, w/v). The results indicate that with increasing of ionic strength by addition of sodium chloride, the extraction recovery was decreased (Fig. 5). It could be related to interaction between chloride and DON that increase the solubility of DON ions in water phase that reduce the affinity of DON in organic phase. Similar results were obtained in previous works [14]. So, further experiments were done without adding salt.

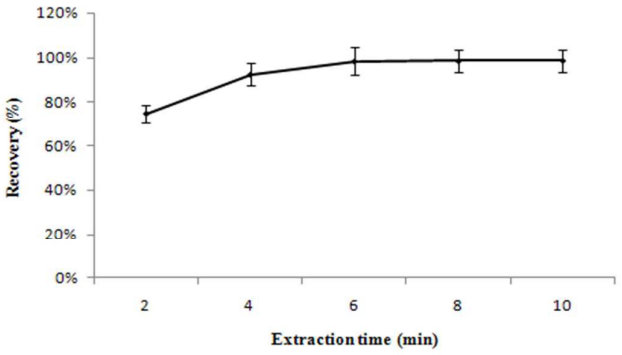

Fig. 3. Effect of extraction time on extraction efficiency (n=3)

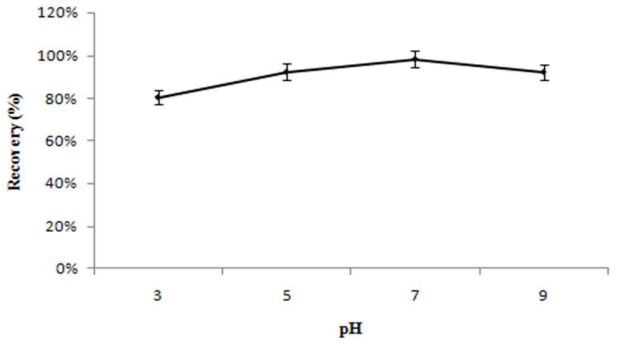

Fig. 4. Effect of pH on extraction efficiency (n=3)

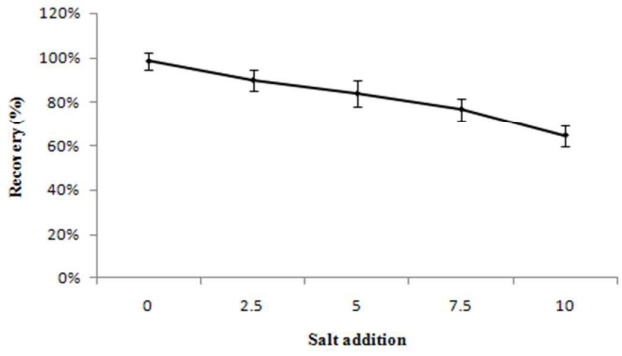

Fig. 5. Effect of salt addition on extraction efficiency (n=3)

Evaluation of Method Performance

A linear calibration curve was obtained between 250–2,000 ng/mL, by dilution of appropriate amounts of DON standard in the final extraction extract obtained under the optimized MNPs extraction conditions; the regression coefficient of matrix match calibration was 0.9985. The LOD and LOQ were calculated based on the standard deviation of the response and the slope of calibration curve[21]; based on the experiments the LOD and LOQ were 45 and 150 µg/kg respectively. The intra and inter day relative standard deviation (RSD) at different levels of spiked samples were calculated, the results indicate that there was no significant statistical difference (t test) between methods (Tab. 1). Analysis of real samples showed that same results were obtained with both methods and none of the samples were contaminated to DON.

Tab. 1. The Intra and inter day recovery and relative standard deviation (RSD) of spiked samples at different levels [a]

				Intra-day (n=3)	Inter-day (n=3)
Analyte	Initial concentration	R^2	Spiking levels (µg/kg)	Mean recovery ± RSD (%)	Mean recovery ± RSD (%)
MNPs	Below LOD	0.9989	500	78.3±5.4	82.7 ± 6.8
			1000	93.1±3.7	86.4 ± 5.7
			1500	90.6±5.9	93.8 ± 7.5
IACs	Below LOD	0.9994	500	85.5±7.2	89.3 ± 4.1
			1000	95.4±1.6	97.2 ± 3.2
			1500	101.2±3.4	98.8 ± 3.7

[a] Extraction conditions: volume of extraction solvents : 800 µL acetonitrile and 250 µL chloroform; the amount of MNPs: 0.05 g; the extraction time: 6 min.

Also, Certified Reference Material (CRM) analysis was done by suggested method. The range of CRM contamination was 2,000±400 µg/kg and mean amount of DON for present procedure was obtained 1,811.2±30.5 µg/kg. The results indicate that the proposed procedure can be used to analysis of DON in wheat samples. The LOD, RSD and recovery of the present method were compared with previous studies including IACs method (Tab. 2). As the LOD data showed, the analytical sensitivity achieved by the proposed method is developed by at least 2 orders of magnitude in comparison with the methods. The RSDs and recovery for the suggested procedure is acceptable and approximately comparable with other methods. In comparison with microextraction methods such as DLLME, CHCl3 and acetonitrile were used for extraction of DON from wheat flour but the LOD and LOQ of the method was higher than the new developed method [13]; this show that Fe_3O_4 MNPs, in addition to reduce of matrix effects could help to improve the extraction recovery. Fe_3O_4 MNPs could be easily removed by using an external magnetic field out of the sample solution and organic solvents, which

this property reduced the time of analysis and easier practical applications. Sunday, et al, reported that gold nanoparticles-dotted 4-nitrophenylazo functionalized graphene (AuNp/ G/ $PhNO_2$) could be used in sensor and they found it sensitive to DON[22], but here, in the proposed procedure, we used Fe_3O_4 MNPs without coating that make the method cheaper and simpler. The chromatograms of the blank and spiked wheat sample are shown in Fig. 6.

Tab. 2. Comparison of the proposed method with other proposed methods including IACs for extraction of DON.

Method	Detection	LOD (µg/kg)	RSD %	Recovery	Ref.
IAC	HPLC-UV	100	3-16	80-100 %	[23]
IAC	HPLC-UV	200	0.9-8.8	86-105%	[24]
IAC	HPLC-UV	250	4.5	91-101 %	[25]
IAC	HPLC-UV	50	3.1	85 %	[13]
DLLME	HPLC-UV	125	1.6	72 %	[13]
Proposed procedure	HPLC-UV	45	3.7-5.8	78-93%	This study

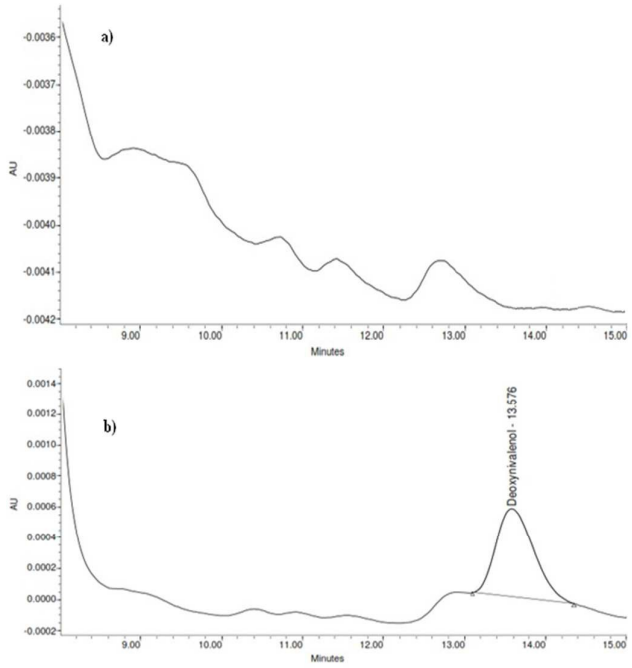

Fig. 6. Typical chromatograms of a) a blank sample and b) spiked sample (1000 µg/kg) of DON after extraction under the optimum conditions

Conclusions

In this study, a new method for the analysis of DON in wheat flour was developed based on MNPs clean-up and mixed organic solvents extraction. The amounts of MNPs affect the purifying performance and the extraction efficiency. The intra- and inter-day relative standard deviation (RSD %) were both $<$ 10%; and the extraction recoveries were comparable with IACs results. As compared with IACs and SPE, the proposed analytical procedure merged clean-up and extraction processes in a single step and offers multiple benefits such as faster analysis, ease of operation, less use of toxic organic solvents and lower cost. So, present method can be used as an alternative method for the determination of DON in wheat flour samples. The validated method was successfully applied for the determination of DON in wheat flour samples, and acceptable results were achieved.

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