Dispersive liquid–liquid microextraction coupled with microfluidic paper-based analytical device for the determination of organophosphate and carbamate pesticides in the water sample

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Abstract
A microfluidic paper-based analytical device (µ-PAD) is a promising new technology platform for the development of extremely low-cost sensing devices. However, it has low sensitivity that might not enable to measure maximum allowable concentration of various pollutants in the environment. In this study, a dispersive liquid–liquid microextraction (DLLME) was developed as a preconcentration method to enhance the sensitivity of the µ-PAD for trace analysis of selected pesticides. Four critical parameters (volume of n-hexane and acetone, extraction time, NaCl amount) that affect the efficiency of DLLME have been optimized using response surface methodology. An acceptable mean recovery of 79–97% and 83–93% was observed at 1 µg L⁻¹ and 5 µg L⁻¹ fortification level, respectively, with very good repeatability (2.2–6.01% RSD) and reproducibility (5.60–10.41% RSD). Very high enrichment factors ranging from 317 to 1471 were obtained. The limits of detection for the studied analytes were in the range of 0.18–0.41 µg L⁻¹ which is much lower than the WHO limits of 5–50 µg L⁻¹ for similar category of analytes. Therefore, by coupling DLLME with µ-PAD, a sensitivity that allows to detect environmental threat and also that surpassed most of the previous reports have been achieved in this study. This implies that the preconcentration step has a paramount contribution to address the sensitivity problem associated with µ-PAD.

Keywords Pesticide · Dispersive liquid–liquid microextraction · Microfluidic paper-based analytical device · Box–Behnken design · Inhibition percent

Introduction
Organophosphate (OP) and carbamates (CM) are the two major categories of pesticides that have been used to increase agricultural productivity [1]. However, their misuse poses serious havoc to the environment as well as a great threat to the health of the user and consumers. This is due to their high toxicity, persistence in soil and water, bioaccumulation in the food chain, mobility, and solubility in water [1–5]. The toxicity of these pesticides is based on the inhibition of acetylcholinesterase (AChE) in insects, fish, birds, and mammals, which allows the neurotransmitter to accumulate at nerve endings, resulting in cholinergic overstimulation [6]. Neurotoxic symptoms, such as headache, excessive salivation, convulsions, respiratory depression, and even death, could be observed. As a result, various government agencies and WHO have set limits on these pesticide residues in waters [7].

Recent research interest in pesticide residue analysis has focused on the development of simple, cost-effective, efficient, and miniaturized techniques that could significantly reduce the use of toxic organic solvents [8–11]. Dispersion liquid–liquid microextraction (DLLME) and microfluidic paper-based analytical device (µ-PAD) are contemporary sample preparation and analysis techniques, respectively, that fulfills the aforementioned requirements.

DLLME is a powerful preconcentration technique that uses a ternary component solvent system. Principally, an extraction solvent (a water-immiscible) is mixed with a dispersive solvent (miscible with both aqueous solvents and extraction solvents) and this mixture is then rapidly injected.
into the aqueous sample (containing analyte of interest). The rapid injection produces a cloudy solution, formed of microdroplets of extraction solvent dispersed in the aqueous sample. Consequently, a large surface area is generated between the aqueous phase and the extraction solvent; thus, analytes are extracted into the extraction phase quickly [8, 12]. However, to maximize extraction efficiency, major operational parameters such as types and volume of extraction and disperser solvents, extraction time, and salting-out effect should be optimized [13, 14]. In the optimization of analytical method, a multivariate method is more desirable than univariate. Because the use of multivariate allows: a dynamic mode (all factors simultaneously changing), to investigate interaction among the factors and a few numbers of experimental runs for the optimization. Several authors optimized DLLME using multivariate method for the extraction organochlorine pesticides [13, 15], OP, and CM pesticide [16–21].

However, with regard to the analysis part, exclusively all the studies employed, very expensive laboratory-based analytical instruments including gas chromatography (GC) [22], high-performance liquid chromatography (HPLC) [23], GC/HPLC–MS, capillary electrophoresis [24], gas chromatography–flame photometric detection [25], and gas chromatography–tandem mass spectrometry [21]. These analytical instruments are complex, and require well-trained technicians and not easily accessible in all laboratories. Alternatively, µ-PADs are a new technology platform for the development of extremely low-cost sensing applications.

Several studies have reported the use of colorimetric µ-PAD for the detection of OP and CM pesticides via AChE inhibition. Badawy and El-Aswad [26] for profenofos and methomyl pesticides; Sankar et al. [27] and Kim et al. [28] for chlorpyrifos; Kavruk et al. [29] for malathion; Apilux et al. [30] for dichlorvos, pirimicarb, carbaryl, and carbofuran; Fernández-Ramos et al. [31] and Jin et al. [32] for carbaryl and chlorpyrifos have demonstrated the use of µ-PAD. For the majority of the studied analytes, high limit of detection (LOD) in the range of 0.065–8.6 mg L−1 had been reported. All the reported LODs are far above the permissible limit set by various regulatory bodies. For example, according to WHO guideline value (GV), the maximum acceptable value/concentration for malathion, chlorpyrifos, carbaryl, and carbofuran are 50, 20, 5, and 5 μg L−1, respectively. This indicates that the sensitivity of the reported µ-PADs are not adequate enough to predict the threshold concentration that might cause environmental threat. Therefore, to overcome this limitation, we used miniaturized DLLME as an enrichment technique to improve the analytical sensitivity of µ-PAD. Furthermore, in this study, multivariate optimization of DLLME for carbosulfan, chlorpyrifos, furathiocarb, and methomyl has been reported for the first time.

**Experimental**

**Chemicals and reagents**

All pesticide standards are analytical grade and were purchased from Sigma-Aldrich (Saint Louis, USA). AChE (Electrophorus electricus, EC 3.1.1.7), Tris (hydroxymethyl) aminomethane, dithiobis-(2-nitrobenzoic acid) (DTNB), and acetylthiocholine iodide (ATChI) were purchased from Sigma-Aldrich (Saint Louis, USA). Bovine serum albumin (BSA) was purchased from Sisco Research Laboratories (Maharashtra, India). HPLC grade, acetone, ethanol, hexane, and methanol were purchased from BIOCHEM Chempharma (ZA Cosne Sur Loire, France). HCl and NaOH were obtained from HiMedia Laboratory (LBS Marg, Mumbai, India).

Tris-buffer stock solution (10 mM) was prepared in distilled water (pH 8.0) and stored at 4 °C. The ATChI stock solution (50 mM) was prepared in Tris-buffer and stored at 4 °C. AChE stock solution of 100 U mL−1 was prepared in Tris-buffer and stored at −20 °C. The working AChE solution was daily prepared by diluting the stock solution using BSA solution. The 50 mM DTNB stock solution was prepared in ethanol and stored at 4 °C. BSA stock solution (1 mg mL−1) was prepared in a Tris-buffer and stored at 4 °C. A standard solution (250 mg L−1) for each pesticide was prepared in methanol and stored at 4 °C in the dark.

**Apparatus**

A wax printer (ColorQube 8580 N, Xerox, CT, USA) was used to print the design. The digital images from µ-PAD were captured using a desktop scanner (Canon PIXMA G2400 Series, Shihen, Vietnam). A drying machine (DHG-9055, Zhengzhou, Henan, China) was used to heat the printed sheets of paper. All sample containers and tools, such as micropipettes and their tips, volumetric flasks, graduated cylinders, spatula, and glassware, were used while working laboratory.

**Experimental design and optimization of DLLME**

The extraction procedure was optimized using Box–Behnken design (BBD). MODDE software version 13.0 (Umetrics, Ume, Sweden) was used to optimize and model the values of independent parameters such as volume of extraction solvent n-hexane (A), volume of dispersive solvent acetone (B), extraction time (C), and NaCl amount (D). During
At the optimization process, carbaryl pesticide was used as a representative pesticide in this study. In each of the four studied variables, high and low set points were selected to design the experiment and were coded at three levels (−1, 0, and +1) corresponding to low, middle, and high levels. Therefore, a total of 27 experimental runs were designed based on the equation \( N = 2k(k-1) + \text{No} \). Where \( N \), \( k \), and \( \text{No} \) represent the total number of experimental runs, the number of factors, and replicate number of central points, respectively (Table S1). Analysis of variance (ANOVA) was used to evaluate the goodness-of-fit determination of the mathematical model to the experimental data using the coefficient of determination (\( R^2 \)) and the adjusted coefficient of determination (\( R^2 \) adj). In addition, Fisher’s F test was used to examine the consistency of the model and the model term was evaluated as a \( p \) value with a 95% confidence level.

**DLLME procedure**

A tape water sample (1000 mL) was collected from Addis Ababa University, Arat Kilo campus and filtered through a 0.45 µm micropore membrane filter. The water sample was stored in a brown glass bottle and refrigerated at 4 °C until the time of extraction. DLLME was carried out under optimized parameters. A 5 mL water sample was taken in 5 mL volumetric flask, and 4.8% (w/v) NaCl was added and then spiked with different standard concentrations of target analytes (one concentration level of a single analyte is spiked at a time).

As it is shown in the schematic procedure of the DLLME, mixture of acetone (0.48 mL) and \( n \)-hexane (47 µL) was injected into the sample solution using a 1.5 mL syringe (gastight, Hamilton, USA) (Fig. 1a). A cloudy solution (water/acetone/\( n \)-hexane) was formed in the solution, and at this stage, the pesticides in the water sample were extracted into fine droplets of \( n \)-hexane (Fig. 1b). After 6 min, more acetone (0.48 mL) was slowly injected into the aqueous bulk as a demulsifier to break the emulsion (Fig. 1c). The emulsion was then separated into two phases in 5–10 s (Fig. 1d). A micro syringe was then used to collect the organic phase (Fig. 1e). The organic phase was dried at room temperature for 6 h and the residue was reconstituted with 50 µL methanol. The reconstituted sample was incubated to 10, 15, 5, 10, and 20 min for carbaryl, carbosulfan, chlorpyrifos, furathiocarb, malathion, and methomyl pesticides, respectively, with AChE, and 1.7 µL of the incubated mixture was added to the detection zone for μ-PAD analysis.

**Fabrication of μ-PADS and colorimetric analysis**

The μ-PAD was designed in the form of circular paper zone of 10 mm diameter using Microsoft Office Power Point 2016 and printed on the sheet of Whatman filter paper (Chromatography Paper 1CHR, Whatman™, GE Healthcare Lifesciences, UK) using a wax printer. The patterned paper was heated at 120 °C for 2 min in a drying machine to melt and permeate the wax ink into the paper matrix, thus forming a hydrophobic barrier [33]. The backside of the device was sealed with scotch tape to prevent loss of loaded reagent and samples (Fig. 1f) during the colorimetric assay.

Multivariate optimized operational parameters from our previous report (1.72 µL of 1 U mL\(^{-1}\) AChE, 2.5 µL of 1 mM ATChI, and 1.4 µL of 1 mM DTNB) were used for the colorimetric assay of the pesticides. The color image
generated by the reaction among AChE, incubated pesticides, DTNB, and ATChI was captured using a desktop Canon Scanner. ImageJ 1.46 s software (National Institutes of Health, USA) was used to analyze the region of interest (ROI) of the images (Fig. 1h). Eight-bit mean greyscale value was set and adjusted based on the gray intensity to yield higher intensity values. Finally, the blue-colored wax background was effectively removed by applying a color threshold window and inverted. The color intensity at a test zone was measured as a mean gray color intensity in the RGB channels.

Method validation

To evaluate the performance of the DLLME, the blank tap water samples spiked with all analytes at different concentrations of pesticides were enriched with DLLME and were finally analyzed by the µ-PADS’ system under optimized experimental conditions. Prior to the spiking procedure, water samples were analyzed and found to be free of pesticide contamination. The enrichment factor (EF) was defined as the ratio of the concentration of analyte in the organic phase ($C_{org}$) to the concentration of the initial analyte in the aqueous sample ($C_{aq}$) and calculated based on Eq. (1) [34, 35]. Linearity was investigated with seven different concentrations levels in the range of 0.625–40 µg L$^{-1}$ for carbaryl, furathiocarb, malathion, and methomyl, and 1.25–40 µg L$^{-1}$ for carbosulfan and chlorpyrifos. Triplicate analyses were performed at each concentration level. The limit of detection (LOD) was calculated as three times standard residuals of the response divided by the slope of calibration curves [36]. The ratio between the amounts of the analytes after spiking ($C_f$) and analyte standard added ($C_a$) was calculated as the percent recoveries as indicated in Eq. (2) [37].

Results and discussion

Selection of extraction and disperser solvent

Low-density solvent, $n$-hexane, was used as an extraction solvent. Miscibility of disperser solvents in both aqueous and extraction solvents is a major requirement for selection. The disperser solvent is used to increase the dispersion of the fine droplets of the extraction solvent in the aqueous solution and enhances the contact area between extraction solvent and aqueous solution, thereby improving the extraction efficiency [38]. It also functions as a demulsifier (terminating solvents) that break up the emulsion formed by destabilizing the interface film between the droplet and water [39]. In this work, acetone, acetonitrile, and methanol were studied as dispersers and terminating solvents. As shown in Fig. 2, acetone extract provided the highest percentage of inhibition, followed by methanol and acetonitrile. Therefore, acetone was selected for further analyses in this study.

Selection of salt type

Adding salt to the sample solution reduces the solubility of the analyte in the aqueous sample and facilitates the transfer of the target analyte from the aqueous phase to the organic solvent phase [40, 41]. In this experiment, NaCl, Na$_2$CO$_3$, and MgSO$_4$ were studied to evaluate the effect of salt on the percentage of inhibition (Fig. 3). Sodium chloride has induced more effective phase separation, for all the analytes studied; hence, it was used as salting-out agent throughout this work.

Multivariate optimization

BBD statistical analysis and the model fitting

The BBD matrix and corresponding factors are shown in Table 1. The polynomial in Eq. (3) depicts the empirical relationship between the percentage of inhibition ($I$) and the four independent variables

$$EF = \frac{C_{org}}{C_{aq}}$$

$$\%R = \left(\frac{C_f}{C_a}\right) * 100.$$
The results of ANOVA for extraction of pesticides are shown in Table 2. A large value of $F$ (85.82) indicates that most of the variables in the response can be explained by the regression equation, and probability values less than 0.05 are considered to be statistically significant. The non-significant value of lack of fit ($p = 2.59 > 0.05$) implies that the quadratic model is suitable for predicting the response and adequately fits with the experimental data. The regression model showed a high correlation (0.98) between the observed and predicted responses (Fig. 4a), suggesting that the proposed model equation yields satisfactory and accurate results. It implies that the predicted values were close to the observed values. The linear, quadratic, and interaction effects of the studied parameters on percentage of inhibition are shown in Fig. 4b. It revealed that the linear effect of the extraction time and volume of $n$-hexane have negative significance, whereas the volume of acetone has positive significant effect. However, the amount of NaCl did not show a significant linear effect. The quadratic and interaction effects of all the studied variables have negative significant effects on the percentage of inhibition except the interaction between the volume of $n$-hexane and acetone.

\[
I\% = -276.1 + 5.127A + 425.8B + 33.15C + 16.655D - 0.03020A^2 - 369.3B^2 - 1.2596C^2 - 0.6499D^2 - 0.247A*B - 0.2968A*C - 0.0796A*D - D - 3.53B*C - 8.23B*D - 0.4456C*D.
\]

The effect of different salt addition to the percentage of inhibition is shown in Fig. 3. Conditions: 50 µL of $n$-hexane; 1.00 mL (0.5 + 0.5 mL) of acetone and extraction time 6 min (1 µg L$^{-1}$ of each pesticide).

**Table 1** Design matrix in the BBD for parameters’ optimization

| Parameters                  | Code | Levels |
|-----------------------------|------|--------|
| Volume of $n$-hexane (µL)   | A    | −1  0  1 |
| Volume of acetone (mL)      | B    | 0.20 0.45 0.70 |
| Extraction time (min)       | C    | 2  6  10 |
| NaCl amount (%/w/v)         | D    | 0  5  10 |

**Table 2** Analysis of variance (ANOVA) for BBD

| Source of variation | Degree of freedom | Sum of the square | Mean square | $F$ value | $p$ value |
|---------------------|-------------------|-------------------|-------------|-----------|-----------|
| Model               | 14                | 7776.99           | 555.50      | 85.82     | 0.000     |
| A                   | 1                 | 160.45            | 160.45      | 24.79     | 0.000     |
| B                   | 1                 | 263.48            | 263.48      | 40.71     | 0.000     |
| C                   | 1                 | 73.46             | 73.46       | 11.35     | 0.006     |
| D                   | 1                 | 12.12             | 12.12       | 1.87      | 0.196     |
| A*A                 | 1                 | 778.33            | 778.33      | 120.25    | 0.000     |
| B*B                 | 1                 | 2841.40           | 2841.40     | 439.00    | 0.000     |
| C*C                 | 1                 | 2166.35           | 2166.35     | 334.70    | 0.000     |
| D*D                 | 1                 | 1407.97           | 1407.97     | 217.53    | 0.000     |
| A*B                 | 1                 | 6.08              | 6.08        | 0.94      | 0.352     |
| A*C                 | 1                 | 2254.35           | 2254.35     | 348.30    | 0.000     |
| A*D                 | 1                 | 253.29            | 253.29      | 39.13     | 0.000     |
| B*C                 | 1                 | 49.98             | 49.98       | 7.72      | 0.017     |
| B*D                 | 1                 | 423.54            | 423.54      | 65.44     | 0.000     |
| C*D                 | 1                 | 317.73            | 317.73      | 49.09     | 0.000     |
| Error               | 12                | 77.67             | 6.47        |           |           |
| Lack-of-fit         | 10                | 72.11             | 7.21        | 2.59      | 0.310     |
| Pure error          | 2                 | 5.56              | 2.78        |           |           |
| Total               | 26                | 7854.66           |             |           |           |
The summary of the model fit plot is depicted in Fig. S1. The result showed that the percent of the variation of the response predicted by the model is $R^2 = 95\%$. This indicates that only 5% of the variation of the responses was not predicted by the model. The model validity result ($0.71 > 0.25$) indicated that there is no lack of fit of the model. Model reproducibility (99%) represents variability in response under the same conditions and is often obtained at center points.

**Response analysis**

The relationship between various independent parameters on the responses is represented with two-dimensional contour plots in RSM (Fig. 5). These plots were obtained for a given pair of factors at fixed and optimal values of other variables. The contour plot in Fig. 5a, b shows the effects of the volume of n-hexane versus the volume of acetone and extraction time. At the lower and higher volume of n-hexane and acetone, and extraction time, the percentage of inhibition of the pesticides is low, which indicated low extractability of the target pesticides. Percentage of inhibition increases as the volume of n-hexane, the volume of acetone, and extraction time increase in the range of 30–47 µL, 0.2–0.48 mL, and 2–6 min, respectively, and started to decrease beyond these ranges. At lower volume of n-hexane and acetone, extraction of the analytes is disturbed due to incomplete dispersion of the organic solvents in aqueous samples. Whereas at high volumes of the organic solvent, polarity of the aqueous sample decreases due to dissolution of disperser and extraction solvents in the aqueous phase that leads to a decrease in partition coefficients of analytes and extraction efficiencies [42]. Figure 5c shows the effect of the volume of acetone versus extraction time. Increasing the extraction time from 2 to 6 min will increase percentage of inhibition as the volume of acetone and the salt amount increases from 0.2 to 0.48 mL and 0 to 4.8% (w/v), respectively, and beyond the 6 min extraction time, the percentage of inhibition decrease. This could be due to long extraction times that may result in the degradation of thermolabile compounds [43, 44] and the possible dissolution of disperser solvent in the sample [45].

**Selection of optimum conditions**

Optimal conditions for the extraction procedure were derived to obtain the maximum percentage of inhibition. RSM selects and predicts the best mode of operation for the range of variables used (Table S2). The model estimated a maximum percentage of inhibition of 86.76% under the optimal condition of 47 µL n-hexane, 0.48 mL acetone, 6 min extraction time, and 4.8% (w/v) NaCl. A validation experiment was carried out under the optimal conditions and yielded 86.58% percentage of inhibition, which is comparable to the model-predicted value of 86.76%. The model also predicts the contribution of each factor in the percentage inhibition to be 17.2, 32.1, 28.1, and 22.6%, for the volume of n-hexane, the volume of acetone, extraction time, and the amount of NaCl, respectively.
Analytical features of DLLME-µ-PADs

Analytical merits of the developed DLLME-µ-PADs method are depicted in Tables 3 and 4. The average recovery was found to be in ranges from 79 to 97% with RSD between 2.21 and 10.46%. EFs for the studied pesticides were in the range of 317–1471. The DLLME method shows good linearity in the range (0.625–40 µg L⁻¹) with a correlation coefficient in the range 0.9968–0.9987. The LODs for carbaryl, carbosulfan, chlorpyrifos, furathiocarb, malathion, and methomyl were 0.19, 0.36, 0.41, 0.17, 0.18, and 0.2 µg L⁻¹, respectively, which is below the maximum residue limits of EU for drinking water.

The performance of DLLME-µ-PADs method was compared to the previously reported µ-PAD method [27, 28, 31, 32] in terms of linear range, LOD, and correlation coefficient. As it can be seen in Table 4, the proposed analytical method has an equivalent or wider linear range and provides better correlation coefficient and lowest LOD value compared with the reported methods. This implies that the proposed method is an efficient and sensitive that can be used to extract/concentrate and measure OP and CM pesticides in water samples.

**Conclusion**

In this study, miniaturized sample preparation (DLLME) and analysis method (µ-PAD) have been coupled to demonstrate a simple, rapid, and sensitive analytical method for the determination of six pesticides in water sample. The DLLME method has been successfully optimized for the preconcentration step. The volume of n-hexane and acetone, and extraction time are found to be the critical operational parameters affecting the DLLME. Under the optimized experimental conditions, the developed method has offered high enrichment factors in the range of 317–1471 and lower LODs ranging from 0.17 to 0.41 µg L⁻¹ for the analyses of trace level of target analytes.

Table 3 Precision and average recovery obtained for water sample

| Pesticides      | 1 µg L⁻¹ Repeatability (%RSD) | 5 µg L⁻¹ Repeatability (%RSD) | 1 µg L⁻¹ Reproducibility (%RSD) | 5 µg L⁻¹ Reproducibility (%RSD) | Recovery (%) 1 µg L⁻¹ | Recovery (%) 5 µg L⁻¹ | EF     |
|-----------------|-------------------------------|-------------------------------|---------------------------------|---------------------------------|-----------------------|-----------------------|--------|
| Carbaryl        | 2.21                          | 3.31                          | 5.60                            | 7.70                            | 85.80                 | 82.15                 | 1421   |
| Carbosulfan     | 5.19                          | 4.41                          | 9.92                            | 6.27                            | 78.50                 | 85.44                 | 472    |
| Chlorpyrifos    | 4.89                          | 5.51                          | 7.58                            | 7.46                            | 86.98                 | 92.46                 | 317    |
| Furathiocarb    | 4.01                          | 5.09                          | 9.94                            | 10.46                           | 90.76                 | 93.17                 | 1471   |
| Malathion       | 2.95                          | 5.72                          | 9.04                            | 8.94                            | 95.34                 | 83.23                 | 1000   |
| Methomyl        | 6.01                          | 4.91                          | 9.94                            | 9.16                            | 97.25                 | 93.31                 | 750    |
Table 4 Comparison of μ-PAD-DLLME with other μ-PAD techniques for the determination of the pesticides

| Methods            | Analyte     | Linear range (µg L⁻¹) | Correlation coefficient | LOD (µg L⁻¹) | References                  |
|--------------------|-------------|-----------------------|-------------------------|-------------|-----------------------------|
| µ-PAD-DLLME        | OP and CM   | 0.625–40              | 0.9968–0.9987           | 0.17–0.41   | This study                  |
| µ-PAD              | Chlorpyrifos| 100–1000              | 0.9980                  | 65          | [27]                        |
| µ-PAD              | Carbaryl    | 25–2500               | 0.9900                  | 29          | [27]                        |
| µ-PAD              | Chlorpyrifos| 1000–100,000          | 0.9600                  | 8600        | [28]                        |
| µ-PAD              | Carbaryl    | 0.24–20               | 0.9930                  | 0.24        | [31]                        |
| µ-PAD              | Chlorpyrifos| 2–45                  | 0.9953                  | 2           | [31]                        |
| µ-PAD              | Carbaryl    | 10–1000               | 0.9951                  | 771         | [32]                        |
| µ-PAD              | Chlorpyrifos| 100–10,000            | 0.9984                  | 253         | [32]                        |

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Declarations

Conflict of interest The authors declare no conflict of interest.

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