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Original Research

Sensitive and multiplexed analysis of aflatoxins using time-of-flight secondary ion mass spectrometry

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Abstract A rapid and reliable analysis of toxins is prerequisite for food control and human healthcare. Here we demonstrate a simple and multiplexed assay of aflatoxins using time-of-flight secondary ion mass spectrometry (TOF-SIMS). By simply adsorbing either a single analyte or mixed ones onto a gold substrate, the corresponding secondary molecular ions ($[M+H]^+$) were clearly observed in a single mass spectrum. As a result of concentration-dependent peak intensity, quantitative and multiplexed analysis of different aflatoxin analogs from corns was accomplished with immunoaffinity column and TOF-SIMS analysis, which showed a good correlation with HPLC data. The detection sensitivity was estimated to be as low as 10 ng mL^{-1}. This approach presented here will find a wide application to detection of low-levels of toxins in a rapid and multiplexed way.

Keywords: Aflatoxin, TOF-SIMS, Quantification, Multiplexed analysis

Introduction

Aflatoxins are toxic secondary metabolites produced mainly by the fungal species such as *Aspergillus flavus* and *Aspergillus parasiticus* that contaminate a wide range of foods and animal feedstuffs stored under temperature and humidity conditions favorable to fungal growth[1,2]. There are different kinds of aflatoxins, and aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1) and aflatoxin G2 (AFG2), are commonly found in both food and feed. These fungal toxins not only have been considered as potent carcinogens that are extremely hazardous to human liver, but also their chronic effects at low levels of exposure have been of great concern because this results in the decrease of host resistance to infectious diseases[3]. Thus, it is essential to develop a rapid and reliable method for detecting the levels of multiple aflatoxins in a variety of foods for human health.

Common established methods for assaying aflatoxins include thin-layer chromatography (TLC)[4], high performance liquid chromatography (HPLC)[5,6], and enzyme-linked immunosorbent assay (ELISA)[7-9]. These methods have excellent sensitivities, but they typically require extensive sample pretreatment and time-consuming operation due to the need of extraction and cleanup procedures. In addition, they lack the ability to perform simultaneous analysis of different kinds of aflatoxins. As an effective approach, the use of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-MS) using a UV-absorbing ionic liquid matrix was reported for multiplex and high-throughput screening of aflatoxins[10]. Nonetheless, it still requires a suitable matrix that might interfere with the accurate analysis particularly when the analyte is at a low concentration. In recent, time-of-flight secondary ion mass spectrometry (TOF-SIMS) has drawn attention because it allows direct surface identification of intrinsic and unlabeled small molecules ($<1{,}000$

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m/*z*) without requiring a matrix. TOF-SIMS has been shown to offer higher surface sensitivity and chemical specificity than LDI- or MALDI-MS[11-14]. For quantitative and sensitive measurement, TOF-SIMS spectra have been successfully combined with other techniques such as radiolabeling, Fourier transform infrared spectrometry (FT-IR), X-ray photoelectron spectroscopy (XPS) or surface plasmon resonance (SPR) for analyzing molecular density on the uppermost layers (10-15Å)[15-19].

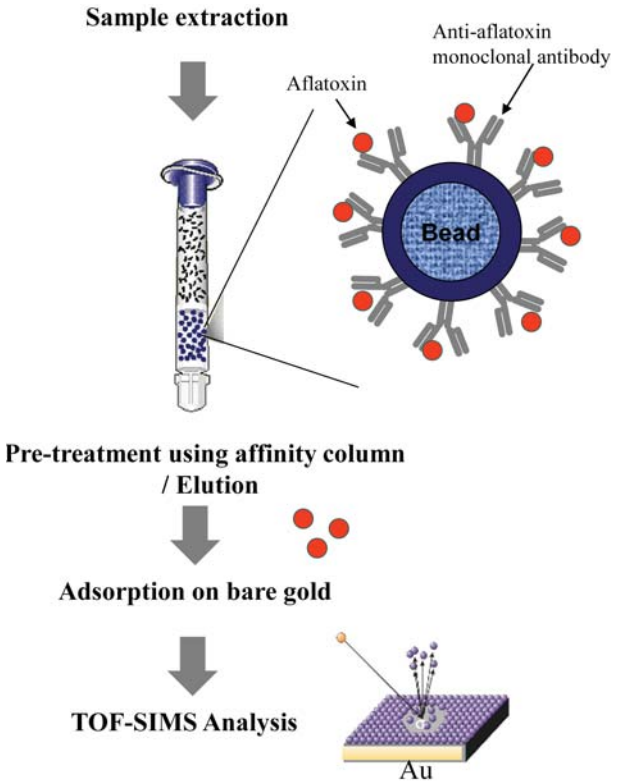
Here we present a simple and multiplexed assay of aflatoxins using TOF-SIMS. Even though TOF-SIMS is widely used in various fields, there have been no studies regarding biosensing of toxins in foods. Following simply adsorption of either a single analyte or mixed ones onto a gold substrate, aflatoxins were subjected to TOF-SIMS analysis. The corresponding secondary molecular ions ($[M+H]^+$) were clearly observed in a single mass spectrum, which enabled a quantitative and multiplexed analysis of different aflatoxin analogs from corns. To validate accuracy in terms of detection sensitivity, mass spectra obtained by TOF-SIMS were correlated with data from HPLC. Details are reported herein.

Results and Discussion

Typical process for assaying aflatoxins using TOF-SIMS is depicted in Scheme 1. As noted, four major steps are included: sample extraction, pretreatment/elution, adsorption and TOF-SIMS analysis. Since the gold substrate greatly enhances the emission of secondary molecular ions of analytes in TOF-SIMS, the accurate identification of the aflatoxin with both qualitative and quantitative information can be attained on gold, which is nearly impossible in fluorescence-based methods.

To get some insight into secondary ion peaks of four aflatoxins, standard aflatoxin solution was directly deposited onto the gold substrate without any purification step, followed by TOF-SIMS measurement. As depicted in Figure 1A, when each aflatoxin was adsorbed onto the gold substrate at a final concentration of 100 ng mL^{-1}, respective secondary molecular ions in TOF-SIMS analysis were distinctly observed for $[M+H]^+$ (*m*/*z* 313.3 for AFB1, *m*/*z* 315.3 for AFB2, *m*/*z* 329.3 for AFG1, and *m*/*z* 331.3 for AFG2). Despite the minor difference in molecular mass of four aflatoxins, this result indicates that the use of TOF-SIMS allows identification of molecular ions at high resolution, whereas this would not be possible with the general MALDI-MS spectra due to matrix-derived interference.

To check the possibility of multiplexed analysis of



Scheme 1. Schematic for analyzing aflatoxins using TOF-SIMS. After pre-concentration and clean-up of aflatoxins from real sample using affinity column, the eluents are directly adsorbed onto gold and subjected to TOF-SIMS analysis.

aflatoxins, four equivalent aflatoxins were mixed and adsorbed onto the gold surface for TOF-SIMS analysis. The final concentration of each aflatoxin was fixed at 100 ng mL^{-1} same as a single spectrum, As shown in Figure 1B, the mixed molecular ions were clearly separated and observed in a single spectrum, although the ion peak intensity is slightly reduced compared to each spectrum in Figure 1A. This result demonstrates that TOF-SIMS analysis enables multiplexed analysis of various aflatoxins at high resolution. Given that the concentration is predictable from peak intensity, it would be possible to quantify aflatoxin species in a single run.

To address whether the aflatoxin is quantitatively analyzed using TOF-SIMS, the different concentrations of AFB1, ranging from 10 ng mL^{-1} to 10 μg mL^{-1} (equivalent to 0.01-10 ppm), were analyzed onto the gold substrate (Figure 2A). When the generated peak intensity was plotted after normalized to that of $[Au_1]^+$ that is constantly produced from the gold substrate, the ion peak increased in proportion to the concentration of aflatoxin (Figure 2B). While three other aflatoxins were subjected to the same analysis, similar calibration

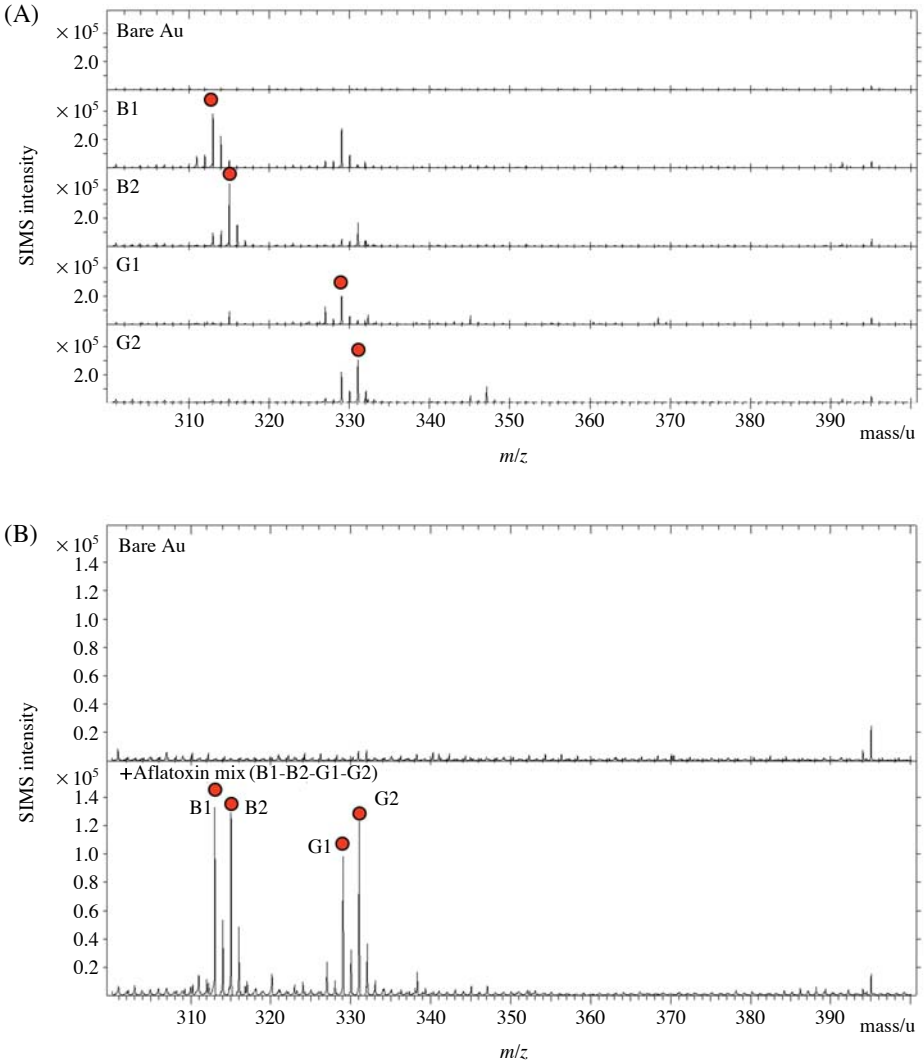


Figure 1. Individual (A) and multiplexed (B) analyses of four aflatoxins (B1, B2, G1, and G2) using TOF-SIMS. The concentration of each aflatoxin was fixed at 100 ng mL^{-1}.

Table 1. Detected molecular ions, dynamic range, linearity (R^2), and LOD for analysis of aflatoxins using TOF-SIMS.

Aflatoxin	$[M+H]^+$ (m/z)	Dynamic range (ng mL^{-1})	Linearity (R^2)	LOD (ng mL^{-1})
Aflatoxin B1	313.3	10-10,000	0.97	10
Aflatoxin B2	315.3	10-10,000	0.96	10
Aflatoxin G1	329.3	10-10,000	0.95	10
Aflatoxin G2	331.3	10-10,000	0.95	10

curves were obtained from each aflatoxin. Limit of detection (LOD) and the dynamic range were similar among respective aflatoxins and were given in Table 1. Although the actual dynamic range might be affected in real food sample, the range was detectable over 3 logs with a good linearity ($R^2 > 0.95$), which is one order of magnitude wider than that acquired by liquid chromatography/atmospheric pressure chemical ionization mass spectrometry (LC/APCI-MS)[20]. The detection sensitivity was estimated to be 10 ng mL^{-1} (10 ppb), which is comparable[21,22] or little poorer than that of using the previous methods[20,23,24] However, considering that other methods were generally performed with concentration steps, the detection limit in this

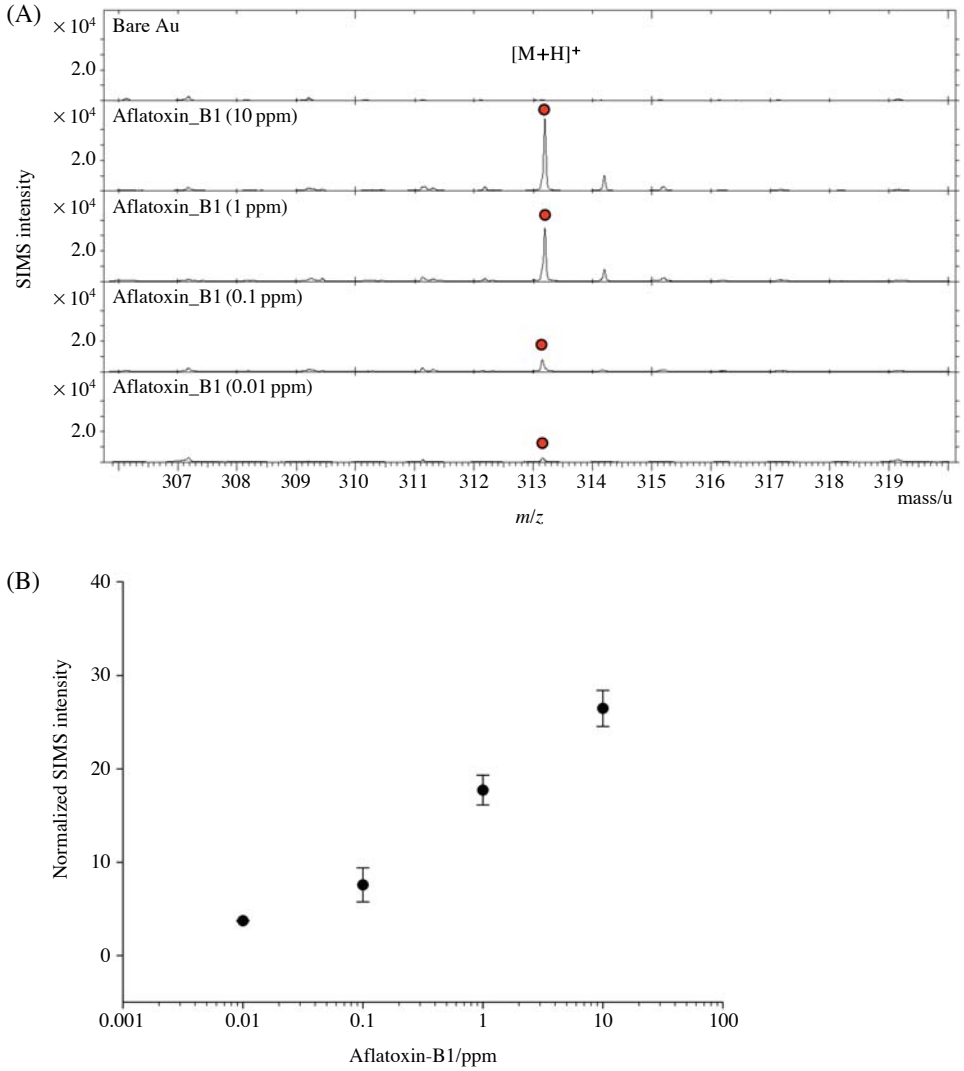


Figure 2. Quantitative analysis of AFB1 using TOF-SIMS. The varied concentrations of AFB1 were analyzed over the range of 0, 0.01, 0.1, 1, and 10 ppm. The resulting peak intensities (A) of molecular ions (*m/z* 313.3) were plotted as a function of AFB1 concentration (B). The standard deviation was obtained from three independent experiments.

study is feasible and expected to detect sub-ppb concentration of aflatoxin if appropriate concentrator is applied. Indeed, it should be noteworthy that high detection sensitivity in real sample can be achievable when immunoaffinity pre-concentration is combined with analyzing tools as demonstrated elsewhere[4,23,25].

In addition to a concentrating step, a cleanup process is also required to remove many interferes that might be abundant in real sample. Unless this step is included, the detection sensitivity would be vulnerable to environmental conditions in relation to salts, detergents and other small toxins. For this, a small cartridge installed with aflatoxin-affinity column was employed as a pretreatment step for TOF-SIMS analysis as shown in Scheme 1. After spiking AFB1 solution at different concentrations (final 1 ppb-1 ppm) into corn extract (10 mL), the mixed extract was transferred to the cartridge, and then the binding, washing, and elution step was subsequently performed to concentrate AFB1 as well as to remove the undesired substances. Considering the volume ratio of analyte to eluent (10 mL : 1 mL) and known recovery rate of the cartridge (82-94%), the concentration was estimated to be 10-fold higher than that at the initial step, which can be covered within the dynamic range of TOF-SIMS analysis. As consistent with this estimation, when different batches of eluents, of which final concentration would be 10 ppb-10 ppm, were directly deposited onto gold and analyzed in TOF

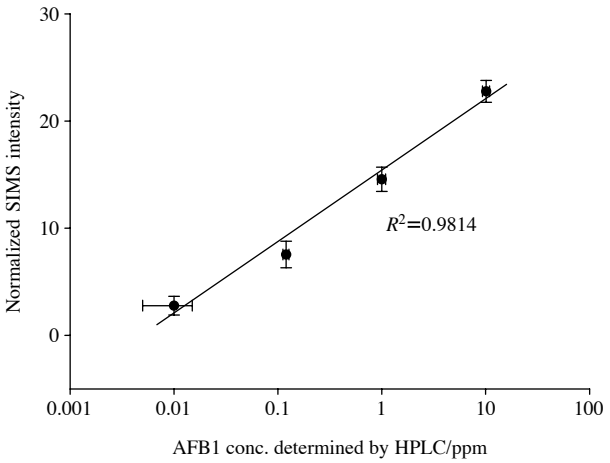


Figure 3. Correlation between TOF-SIMS and HPLC data of low-level spikes for AFB1 from corns. The affinity-based mini-column was used for cleanup and concentration of AFB1. The eluent from mini-column was directly deposited onto gold for TOF-SIMS analysis and was also subjected to HPLC analysis. For TOF-SIMS, analyzing surface area was $500 \times 500\ \mu m^2$.

-SIMS, the molecular ions were successfully observed, showing a similar linearity with that in Figure 1B. This result means that aflatoxins in real sample could be easily detected using TOF-SIMS by combining with simple affinity column treatment. In particular, when the same batches were independently analyzed in HPLC and compared to the quantitative data of TOF-SIMS, a reliable correlation (R^2=0.9814) was found over the tested range (Figure 3). This result obviously shows that TOF-SIMS analysis presented here offers reliable and quantitative analysis of aflatoxin in real sample.

In terms of assaying time, the overall process including cleanup (7-10 min), surface adsorption (60 min) and TOF-SIMS analysis (10 min) appears to take 77-80 min, but ion peak intensity was saturated after 10-min adsorption on gold (data not shown). Especially, if the smaller analyzing area ($100 \times 100\ \mu m^2$) are applied for TOF-SIMS analysis, the analyzing time could be reduced to approximately 20 min, which is much more advantageous over the conventional methods that require more than 2 hours for aflatoxin analysis. Most importantly, multiplexed analysis of aflatoxins can be accomplished in a single run with the widest quantitation range available. Furthermore, TOF-SIMS analysis provides a high resolution for low-level of toxins. Besides the expensive gold substrate, it has been reported that gold nanoparticles could be applied on a conventional glass slide[26,27]. This may allow the assay format to be simple and versatile for the general use.

In conclusion, we have demonstrated a simple and multiplexed assay of aflatoxins using TOF-SIMS. Unlike the conventional methods using TLC or HPLC which require laborious and time-consuming process, TOF-SIMS enables the straightforward analysis of different aflatoxins with low level within a short time. In addition, the ability to detect the toxins in both quantitative and multiplexed ways may contribute to high-throughput analysis of aflatoxins in complex samples. Our strategy, therefore, is anticipated to be applied for developing simple and multiplexed biosensors to detect even very low levels of environmental or pathogenic toxins.

Materials and Methods

Materials

Reagents were purchased from commercial sources: aflatoxin B1, B2, G1, and G2 (Sigma-Aldrich), immunoaffinity column, AflaTest™ (Vicam). All other reagents used were of analytical grade.

Aflatoxin extraction from corns

Corns obtained in Korea were ground using vertical cutter mixer and 50 g of ground corns was placed in a blender jar containing 5 g of NaCl. After adding 100 mL methanol/water (80 : 20, v/v) to the jar and the sample was blended at high speed (1,500 × g) for 1 minute. The extract sample was then divided into five glass bottles and the different concentrations of aflatoxin B1 were additionally added to the extract solution in each glass to give a final concentration of 0, 1, 10, 100, and 1,000 ppb. 10 mL of the mixed solution was applied to a fluted filter paper.

Immunoaffinity column treatment

An aliquot of 10 mL of the aqueous filtered extract was transferred to an AflaTest™ cartridge column at a flow rate of 1-2 drops s^{-1}. The cartridge was rinsed with 10 mL of water. AFB1 was then eluted with 1 mL of methanol, at a flow rate of 1-2 drop s^{-1}. The yellow drops that eluted early were discarded, and the colorless methanolic extract was directly adsorbed to the gold substrates for 60 min and then the surface was dried under a gentle stream of N_2. The other portion of the eluent was subjected to HPLC analysis.

Sample preparation

A gold substrate was prepared by sequentially evaporating a 20Å thick film of Ti and a 400Å thick film of gold onto a Si wafer. Prior to the deposition of the monolayer, the substrate was cut into 10 mm × 10 mm

pieces and cleaned for 5 min by immersion into a piranha solution (1 : 4) 30% H_2O_2: concentrated H_2SO_4 (v/v). *Caution*: the piranha solution reacts violently with most organic materials and must be handled with extreme care. For quantification of aflatoxins, different concentrations of aflatoxin solutions (1, 10, 100, 1,000, and 10,000 ng mL^{-1}) in organic solvent (acetone : methanol =4 : 1, v/v) were adsorbed onto the gold substrates for 60 min at room temperature, followed by a thorough washing with absolute methanol and water. The resultant gold substrates were dried under a stream of N_2 and subjected to TOF-SIMS analysis. Four different aflatoxins (B1, B2, G1, and G2) were independently treated onto gold under the same conditions for quantitative study. For preparation of a mixed working standard solution of aflatoxins, equally mixed sample of four aflatoxins (B1 : B2 : G1 : G2=1 : 1 : 1 : 1, w/v) (each at a final concentration of 100 ng mL^{-1}) was incubated onto gold for 60 min, rinsed with distilled water, dried under a gentle stream of N_2, and subjected to TOF-SIMS analysis.

TOF-SIMS analysis

Ion spectra measurements by TOF-SIMS were carried out with a TOF-SIMS V instrument (ION-TOF GmbH, Germany) using a 25 keV Bi_1^+ primary ion beam source. The ion currents were measured to be 0.5 pA (Bi_1^+) at 5 kHz using a Faraday cup located at the grounded sample holder. A pulse with of 0.7 ns from the bunching system resulted in mass resolution exceeding $M/\Delta M=10^4$ (full width at half maximum [FWHM]) at $m/z > 500$ in the positive mode. The analysis area ($500 \times 500\ \mu m^2$) was randomly rastered by the primary ions, and the primary ion dose was maintained below 10^{12} ions/cm^2 to ensure static SIMS conditions. Positive ion spectra were internally calibrated by using the H^+, H_2^+, CH_3^+, $C_2H_3^+$, and $C_3H_4^+$ signals.

HPLC analysis

An Alliance 2690 HPLC system (Waters, U.S.A.), including a binary high-pressure gradient pump, an automatic sample injector, a column thermostat, and a photodiode array, was used. Separation of aflatoxins was performed in a Waters XTerra (U.S.A.) reverse phase C18 column, 5 μm particle size, 250 × 4.6 mm i.d. A specific Nova-Pack guard column was placed between the autoinjector and column. Chromatographic separation was conducted in a mobile phase at a flow rate of 1 mL/min with solvents containing methanol : water (45 : 55). Detection was carried out using a wavelength program with excitation and emission wavelengths of 360 and 418 nm, respectively.

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