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RESEARCH ARTICLE

# Production and purification of ochratoxin A: effects of media, light, and fungal strain variety

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**Abstract**

Ochratoxin A (OTA) is a mycotoxin produced by fungal species from the *Aspergillus* and *Penicillium* genera, frequently contaminating food products such as cereals, coffee, and dried fruits. OTA's nephrotoxic, immunosuppressive, and potentially carcinogenic properties pose significant risks to food safety and public health. Efficient and reproducible protocols for OTA production and purification are essential to facilitate downstream applications, particularly in toxicological studies where high-purity OTA is required. This study investigated the effects of growth media, light conditions, and fungal strain variability on OTA production. Six different liquid media were first evaluated using five fungal strains to determine the optimal medium for OTA biosynthesis. The two highest-yielding strains from this screening were subsequently tested under different light conditions and time points to optimise production parameters. These optimised conditions were then applied to a large-scale screening of 53 fungal strains to assess their OTA production capabilities. Results showed that Czapek yeast autolysate medium yielded the highest OTA concentrations, with strains of *Aspergillus westerdijkiae* and *Aspergillus steynii* emerging as the highest-yielding producers. Continuous darkness significantly enhanced OTA production, with peak yields observed between days 14 and 17. The purification protocol achieved a near 100% purity level with a final recovery efficiency of 75%, ensuring a reproducible method for obtaining high-purity OTA. The optimised conditions are compatible with future isotope labelling applications, where defined media are essential. These findings contribute to standardising OTA production and purification for toxicological studies, metabolomics, and use as analytical standards.

**Keywords**

ochratoxin A production – *Aspergillus steynii* – liquid-liquid extraction – solid-phase extraction – preparative HPLC

## 1 Introduction

Ochratoxin A (OTA) is a secondary metabolite produced by several species of fungi, most notably within the genera *Aspergillus* and *Penicillium* (Alshannaq and Yu, 2017). OTA was first isolated in 1965 as a fungal metabolite of *Aspergillus ochraceus*, exhibiting toxic effect on animals (Van Der Merwe *et al.*, 1965a,b), but it has

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since been identified in various other species (Ostry *et al.*, 2013). OTA is widely found in various agricultural products, particularly cereals, coffee beans, dried fruits, spices, and wine (Ben Miri *et al.*, 2024; Schrenk *et al.*, 2020). Its prevalence in agricultural commodities, especially under high humidity and improper storage conditions, poses a persistent challenge to food safety and public health (Gruber-Dorninger *et al.*, 2019).

OTA is known for its toxicity in animals and humans, with nephrotoxicity (kidney damage) being the most documented effect (Boorman *et al.*, 1992). Chronic low-level exposure is particularly concerning, especially in regions with weak food safety regulations (Schrenk *et al.*, 2020). OTA has been linked to Balkan Endemic Nephropathy (BEN), a chronic kidney disease prevalent in Eastern Europe (Castegnaro *et al.*, 2006; Grollman and Jelaković, 2007; Pfohl-Leszkowicz and Manderville, 2007), although the causal link between OTA and BEN remains disputed, as some studies highlight inconsistencies and alternative explanations for the disease aetiology (Bui-Klimke and Wu, 2014). Additionally, OTA exhibits immunosuppressive, hepatotoxic, and genotoxic effects, raising concerns about its potential carcinogenicity (Creppy, 1999; Haubeck *et al.*, 1981; Shin *et al.*, 2019). The International Agency for Research on Cancer (IARC) classifies OTA as a group 2B carcinogen (possibly carcinogenic to humans (IARC, 1993)).

OTA can persist even after cooking, drying, or fermentation, making it difficult to eliminate once contamination has occurred (Milani and Maleki, 2014; Scott *et al.*, 1995; Scudamore *et al.*, 2003). Given its toxicity and stability, regulatory bodies have imposed strict limits on OTA in food products. The European Union (EU) enforces maximum allowable OTA levels for different food categories, with limits ranging from 0.5 μg/kg in baby food to 5 μg/kg in unprocessed cereals, 2-5 μg/kg in coffee and wine, 8 μg/kg in dried vine fruits and wheat gluten, and up to 80 μg/kg in liquorice extract (EC, 2023). In contrast, federally mandated limits are lacking in the US, where only general food safety guidelines are advised by the Food and Drug Administration (FDA) (FDA, 2024). Despite these measures, OTA contamination remains widespread, particularly in developing countries, where storage conditions are suboptimal and monitoring efforts are limited (Banahene *et al.*, 2024; Nazareth *et al.*, 2024). Several studies have indicated that OTA can also be found in dry-cured meat and in ripened cheese, primarily due to environmental contamination that promotes fungal growth on the surface. (Anelli *et al.*, 2019; Dall'Asta *et al.*, 2010; Pattono *et al.*, 2013; Rodríguez *et al.*, 2024; Sakin *et al.*, 2018)

Optimal OTA production generally occurs under warm temperatures (typically between 20-30 °C) and high humidity levels (70-90%), conditions commonly found in tropical and subtropical regions (Ben Miri *et al.*, 2024). However, OTA production capacity varies significantly among species and even among different strains of the same species. For example, *Penicillium verrucosum* is a major OTA producer in cooler climates (Kuruc *et al.*, 2015), while *Aspergillus* species are more prevalent in warmer regions, thriving in cereals and other commodities stored under hot, moist conditions (Bellí *et al.*, 2005; Esteban *et al.*, 2006; Ramos, 1998). This variability makes it essential to study multiple strains under various conditions to fully understand the potential for OTA contamination. Despite the known role of *Aspergillus* and *Penicillium* in OTA production, many studies have focused on only a small number of fungal species or strains (Esteban *et al.*, 2006; Ramos, 1998; Zhang *et al.*, 2024). This limited scope overlooks the diversity in OTA production potential among strains, creating a need for comprehensive screening under varied culture conditions.

OTA is most commonly produced in solid-state fermentations using cereal-based substrates such as wheat, rice, or maize. These complex media support high yields, typically in the range of hundreds to thousands of milligrams per kilogram of substrate, and are well established for bulk toxin production (Bunge *et al.*, 1979; Lindenfelser and Ciegler, 1975; Wawrzyniak and Waśkiewicz, 2014). However, they consist of undefined components like starch, lignin, and cellulose, for which isotope labelled versions are either unavailable or unaffordable. This makes them unsuitable for applications requiring uniformly labelled OTA. Synthetic liquid media with a defined composition, in contrast, can be formulated exclusively from defined components, making them compatible with labelling experiments. The trade-off is significantly lower OTA yields, typically in the range of tens to low hundreds of milligrams per litre and greater sensitivity to conditions like carbon source repression and trace nutrient availability (Davis *et al.*, 1972; Koteswara Rao *et al.*, 2013; Meca and Ritieni, 2009).

The objectives of this study were threefold. First, to identify fungal strains and media compositions that yield high OTA levels in semi-synthetic liquid media. Second, to optimise growth parameters, including light and incubation time. Third, to establish a scalable, stepwise purification protocol for obtaining OTA of sufficient purity for toxicological and metabolomics applications. Although no isotope labelling was performed

TABLE 1 Composition of media

| | BMEAM[1] | CYA[1] | FM[2] | FMM[3] | MC[4] | YES[4] |
|---|---|---|---|---|---|---|
| sucrose [g/l] | 30 | 30 | 30 | 30 | 30 | 40 |
| $NaNO_3$ [g/l] | - | 3 | - | 2 | - | - |
| L-glutamic acid [g/l] | - | - | 10 | - | - | - |
| urea [g/l] | - | - | - | - | 3 | - |
| yeast extract [g/l] | - | 5 | - | - | - | 20 |
| peptone [g/l] | 1 | - | - | - | - | - |
| malt extract [g/l] | 40 | - | - | - | - | - |
| corn steep liquor [g/l] | - | - | - | - | 0.5 | - |
| KCl [g/l] | - | 0.5 | - | 0.5 | 0.5 | - |
| $KH_2PO_4$ [g/l] | - | 1 | 1 | 1 | 1 | - |
| $MgSO_4 * 7H_2O$ [g/l] | - | 0.5 | 0.5 | 0.5 | 0.5 | - |
| $CuSO_4 * 5H_2O$ [mg/l] | 5 | 0.5 | 0.5 | 0.5 | 0.5 | - |
| $ZnSO_4 * 7H_2O$ [mg/l] | 10 | 10 | 10 | 10 | 10 | - |
| $MnSO_4 * H_2O$ [mg/l] | - | 0.1 | 0.1 | 0.1 | 0.1 | - |
| $Fe(NH_4)_2(SO_4)_2 * 6H_2O$ [mg/l] | - | 2 | 2 | 2 | 2 | - |
| $H_3BO_3$ [mg/l] | - | 0.1 | 0.1 | 0.1 | 0.1 | - |
| $Na_2MoO_4 * 2H_2O$ [mg/l] | - | 0.1 | 0.1 | 0.1 | 0.1 | - |

1 Koteswara Rao *et al.* (2013).
2 Wei *et al.* (1971).
3 Leslie and Summerell (2006).
4 Lillehoj *et al.* (1978).

here, the protocol was designed to support future isotope incorporation. This work provides a practical and reproducible approach for producing OTA in liquid culture and a flexible purification strategy that can be adapted depending on the required purity level and available resources.

## 2 Materials and methods

### *Fungal strains and culture conditions*

Fungal strains

A total of 53 *Aspergillus* and *Penicillium* strains from species shown to produce OTA (Wang *et al.*, 2018) were used in this study (Supplementary Table S1). The strains were obtained from the following strain collections: (1) The former Austrian Center of Biological Resources and Applied Mycology (which is still accessible by BOKU researchers, https://boku.ac.at/en/bioconversion/partner/#c72521), (2) the ITEM collection (https://item.bio-aware.com/) and (3) and strains kindly provided by Mycolab (https://mycolab.at/en/). Spores of the strains were stored as freezer cultures in 25% glycerol at –80 °C. For experimental use, strains were activated on potato dextrose agar (PDA) plates and maintained at 4 °C for up to two months. PDA plates were prepared using commercially available Difco™ PDA powder (Becton, Dickinson and Company, Sparks, MD, USA), 39 g were dissolved in 1 L distilled water, autoclaved at 121 °C for 20 minutes, and poured into sterile Petri dishes.

Spores for inoculation were collected by washing the surface of the PDA plates with 2 ml of sterile distilled water and transferring the suspension into Eppendorf tubes. If necessary, the spore suspension was filtered through glass wool to remove residual mycelium. Spore concentrations were determined using a Thoma counting chamber and adjusted to the desired concentration with distilled water. The list of all strains screened for OTA production is given in Supplementary Table S1.

Liquid media

Six different liquid media were used in this study: Blakeslee malt extract autolysate medium (BMEAM), Modified Czapek yeast autolysate (CYA) medium, Modified Ferreira medium (FM), Fusarium minimal medium (FMM), Modified Czapek solution (MC) and Yeast extract-sucrose (YES) medium. The composition of the media is presented in Table 1. Media were sterilised by autoclaving at 121 °C for 20 min.

TABLE 2 List of monitored transitions[1]

| Name | Q1 Mass [Da] | Q3 Mass [Da] | DP [V] | CE [eV] |
|---|---|---|---|---|
| $OTA_1$ (Quantifier) | 404.0 | 239.0 | 90 | 40 |
| $OTA_2$ (Qualifier) | 404.0 | 221.0 | 90 | 40 |
| $OTA_3$ | 404.0 | 102.0 | 90 | 105 |
| $OTB_1$ (Quantifier) | 370.1 | 205.0 | 86 | 35 |
| $OTB_2$ (Qualifier) | 370.1 | 103.1 | 86 | 80 |

1 DP = declustering potential; CE = collision energy; OTA = ochratoxin A; OTB = ochratoxin B.

Growth conditions and sampling

20 ml of the respective media in magenta boxes were inoculated with approximately $10^5$ fungal spores, yielding a final concentration of 500 spores/ml, and incubated at 25 °C in continuous darkness or under a 16 h light/8 h dark cycle, respectively. Cultures were harvested after 7, 11, 14, 17, or 21 days, respectively, as indicated in the respective experiment. If immediate processing was not possible, cultures were frozen at –20 °C at the respective harvest time point for later analysis.

Additionally, for cultures grown in CYA medium, volumes of 30, 40, and 50 ml were inoculated with proportional amounts of spores to maintain the same final concentration of 500 spores/ml and incubated under the same conditions.

OTA extraction and preparation for measurement

OTA concentration was measured using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) following extraction and appropriate dilution. In cases where cultures had been frozen, they were first thawed at room temperature before transferring the media containing mycelia to 50 ml polypropylene tubes. After incubation the approximate volume of the culture was determined and an equal volume of distilled water was used to rinse the magenta boxes, and then added to the polypropylene tubes to ensure a 1:1 dilution. The tubes were then shaken at 180 rpm for 10 min at 20 °C to thoroughly mix the solution and enhance OTA extraction into the liquid phase. In addition to washing the magenta boxes with distilled water, we also tested protocols where water was added directly to the polypropylene tubes without prior box rinsing, as well as corresponding approaches using methanol instead of water.

The mixture was centrifuged at 3,220 RCF for 10 min to remove the mycelium from the medium, then 1 ml of the supernatant was transferred into 1.5 ml polypropylene safe-lock test tubes. If necessary, samples were centrifuged again at 11,200 RCF to remove any remaining spores or mycelial fragments. The clarified supernatant was then subjected to two dilutions at 1:20, resulting in a final dilution of 1:40 and 1:800, to ensure the OTA concentration was within the linear range for the LC-MS/MS method.

LC-MS/MS method

LC-MS/MS analysis was performed using an Agilent 1100 HPLC system with a DAD detector (Waldbronn, Germany), coupled with a Sciex Triple Quad 3500 mass spectrometer (Framingham, MA, USA). The separation was achieved using a Phenomenex Kinetex Core-Shell C18 Column (2.1 × 50 mm, 2.6 μm particle size; Aschaffenburg, Germany). A gradient of methanol and water (each containing 0.1% formic acid) was used as the mobile phase, with a flow rate of 0.35 ml/min. The gradient started with 50% aqueous methanol and increased linearly to 100% methanol over the course of six minutes. It was then held at 100% methanol for three minutes before returning to the initial conditions for an additional three minutes to allow for column equilibration. This gradient enabled efficient separation of OTA and ochratoxin B (OTB) within a total run time of 12 minutes. The injection volume was 1 μl, and separations were carried out at 35 °C

For mass spectrometric detection, OTA and OTB were monitored using selected reaction monitoring in negative ion mode. The list of monitored transitions is given in Table 2. The electrospray ionisation parameters were electrospray voltage –5,500 V, source temperature 550 °C, curtain gas 25 psi, collision gas 6 psi and ion source gases 1 and 2 at 50 psi each. The calibration curves for OTA and OTB were generated using standard solutions (Biopure™, Romer Labs, Tulln, Austria). The limit of quantification (LOQ) was determined to be 1 μg/l for both compounds.

### *OTA purification protocol*

OTA purification consisted of liquid-liquid extraction (LLE), solid-phase extraction (SPE), and preparative HPLC performed in sequence.

#### Liquid-liquid extraction

Culture filtrates were acidified to pH 3 using formic acid before extraction. Six solvents were tested: heptane, petroleum benzine, ethyl acetate, toluene, dichloromethane, and chloroform. In small-scale experiments, 1 ml of solvent was mixed with 1 ml of culture filtrate in 5 ml polypropylene safe-lock test tubes, vortexed for one minute, and subsequently allowed to separate into organic and aqueous phases. Each condition was tested in triplicate. The aqueous and organic layers were collected and analysed via LC-MS/MS. For large-scale extractions, 500 ml of acidified culture filtrate was extracted with 500 ml of ethyl acetate in a separatory funnel and shaken for five minutes. The organic phase was collected and subjected to a second LLE step using 500 ml of 10 g/l sodium carbonate solution to transfer OTA back into the aqueous phase. The aqueous phase was then acidified to pH 3 with 1 M aqueous HCl before further purification.

#### Solid phase extraction

Strata-X 33 µm polymeric reversed-phase cartridges (5 g/60 ml, Phenomenex) were conditioned with 50 ml methanol, followed by 50 ml of 10% methanol. The 500 ml aqueous OTA solution from the LLE step was loaded onto the cartridge. The cartridge was washed using 50 ml of 50% methanol, and OTA was eluted using 100 ml of acidified methanol (0.1% formic acid in methanol). The eluate was concentrated to 10 ml using a rotary evaporator.

#### Preparative HPLC

Preparative HPLC was performed using an Agilent 1100 HPLC system equipped with a Phenomenex Gemini NX-C18 column (150 mm × 21.2 mm, 5 µm). A gradient of methanol and water (each containing 0.1% formic acid) was used as the mobile phase, with a flow rate of 16 ml/min. The gradient started with 60% aqueous methanol and increased linearly to 100% methanol over the course of 10 minutes. It was then held at 100% methanol for two minutes. An injection volume of 900 µl was used. Detection was performed using a UV/Vis detector set at 220 nm and 325 nm, as well as an evaporative light scattering detector (ELSD). Fractions were collected using peak-based triggering, with an absorbance threshold of 100 mAU and a slope value of 20 mAU/s to define the start and end of collection. Only fractions corresponding to the OTA peak were retained. Collected fractions were freeze-dried to obtain a dry solid.

#### Purity assessment of ochratoxin A

Purity assessment was conducted at each purification step using LC-MS/MS and LC-UV detection. OTA concentration was determined via LC-MS/MS, after which 1 ml of the sample solution was evaporated to dryness at 110 °C overnight. The resulting residue was weighed using an analytical balance, and the measured mass was compared to the theoretical mass of OTA, calculated from the LC-MS/MS determination. Purity was assessed by comparing the actual weight of the dried residue to the expected OTA mass. All measurements were performed in triplicate.

For additional verification, LC with UV detection was performed. While the same analytical column was used as described above, the mobile phases were water and acetonitrile (each containing 0.1% formic acid). The gradient started at 5% aqueous acetonitrile, increased linearly to 100% acetonitrile over 6 min. It was then held at 100% acetonitrile for three minutes before returning to the initial conditions for an additional 3 min to allow for column equilibration. A flow rate of 0.4 ml/min was used. 5 µl of the purified OTA solution was injected. UV signals were recorded at 210, 230, 250, 270, and 290 nm with a 20 nm bandwidth in addition to the MS signal. Additional peaks at 210 nm were noted as indicators of impurities.

## 3 Results

### *Media optimisation*

First, we tested OTA production by six selected strains from the ACBR and ITEM collections. These include two *A. westerdijkiae* strains (MA 5454 and ITEM 18008), two *A. welwitschiae* strains (ITEM 7097 and ITEM 6142), *A. steynii* (ITEM 17426) and *Penicillium nordicum* (EL 145). The following liquid media were tested: BMEAM, CYA, FM, FMM, MC, and YES. For each condition, 20 ml of medium was inoculated with $10^5$ spores and incubated for 14 days at 25 °C in continuous darkness. All experiments were conducted in triplicate.

Significant variations in OTA yields were observed across both media and strains. As shown in Table 3, three of the six strains tested yielded the highest OTA concentrations in CYA medium. In this medium ITEM 17426 produced an average of 425 mg/l. The other two

TABLE 3 Ochratoxin A concentrations (mg/l) in different media across six fungal strains

| Strain number | BMEAM | CYA | FM | FMM | MC | YES |
|---|---|---|---|---|---|---|
| MA 5454 | 33.5 ± 6.71 | 151 ± 16 | 72.7 ± 13.2 | 0.81 ± 0.23 | 69.2 ± 8.6 | <LOQ[2] |
| ITEM 7097 | 14.8 ± 1.24 | 1.47 ± 0.07 | 0.74 ± 0.23 | 0.14 ± 0.01 | 0.08 ± 0.02 | 1.01 ± 0.17 |
| ITEM 6142 | 0.22 ± 0.04 | 0.28 ± 0.06 | 0.11 ± 0.02 | 0.01 ± 0.00 | 0.03 ± 0.01 | 0.82 ± 0.36 |
| ITEM 18008 | 48.8 ± 14.9 | 192 ± 8.12 | 98.6 ± 24.1 | 0.94 ± 0.40 | 86.1 ± 9.3 | 6.58 ± 1.32 |
| ITEM 17426 | 134 ± 50 | 425 ± 12.87 | 65.4 ± 3.4 | 14.1 ± 5.2 | 138 ± 27 | 34.5 ± 4.16 |
| EL 145 | 0.52 ± 0.34 | 0.15 ± 0.10 | <LOQ[2] | <LOQ[2] | 0.07 ± 0.04 | 0.01 ± 0.00 |

1 The highlighted cells indicate the media in which each fungal strain produced the highest amounts of OTA.
2 LOQ = 1 μg/l.

strains, ITEM 18008 and MA 5454, also showed significantly higher OTA levels in CYA than in the other media investigated.

BMEAM and MC media yielded moderate OTA concentrations for some strains, with ITEM 17426 again being the most productive strain. In contrast, FM and FMM media led to generally low OTA production across all strains, often close to or below 1 mg/l.

The YES medium showed minimal OTA production, with most strains either producing low levels or amounts lower than the LOQ.

Inoculating 20, 30, 40, and 50 ml of CYA medium with the same spore concentration (500 spores/ml) resulted in similar OTA concentrations, with no significant variations observed between the tested volumes (Supplementary Table S2). This suggests that larger culture volumes per magenta box can be used for large-scale OTA production without compromising yield.

To evaluate the impact of different extraction approaches on OTA recovery, cultures were processed using four variations: washing or not washing the magenta boxes with either water or methanol after decanting the cultures. All experiments were conducted in triplicate. For both water and methanol, OTA concentrations were consistently lower when the magenta boxes were not washed prior to adding the extraction solvent. Additionally, the no-wash conditions showed greater variability between replicates. In contrast, when the magenta boxes were washed before extraction, OTA concentrations were more consistent across replicates, indicating improved reproducibility. No significant differences were observed between using water or methanol for washing (Supplementary Table S3), suggesting that both solvents are suitable for this purpose.

### *Time and light condition*

Next, we compared OTA production in CYA medium under two different light regimes: continuous darkness and 16 h light/8 h dark. For this, the two strains performing best in the previous experiment were used. ITEM18008 and ITEM17426 were grown under the two light conditions for 7, 11, 14, 17 and 21 days. OTA concentrations over time under both light conditions are illustrated in Figure 1, demonstrating significantly higher OTA production under continuous darkness compared to the light/dark cycle.

In both strains tested, OTA concentration under continuous darkness shows a steady increase, with peak concentrations observed around day 14-17. Specifically, the OTA concentration reached approximately 140 mg/l (ITEM 18008) at day 21 after inoculation, while ITEM 17426 reached a concentration of 370 mg/l already by day 17. In contrast, OTA levels under the 16 h light/8 h dark cycle remained consistently lower, with only minimal fluctuations over time, reaching a maximum of about 20 mg/l (ITEM 18008) and 200 mg/l (ITEM 17426).

### *Strain screening*

A total of 53 fungal strains (Supplementary Table S1) were screened for OTA production under optimised conditions. The fungal species were selected based on a previously published study that lists species reported to produce OTA (Wang *et al.*, 2018). Based on this list, we selected strains available in our strain collections. Screening was conducted in CYA medium at 25 °C under continuous darkness for 14 days. The negative control, consisting of pure culture medium without fungal inoculation, showed no signs of fungal growth and no detectable OTA, confirming the absence of background contamination. The strains displayed a broad range of OTA production capacities and were categorised as follows (see also Supplementary Table S1): 22 strains were non-producers (below the LOQ), 17 were low producers (below 1 mg/l), six were moderate producers (1-20 mg/l), and five were high producers, exceeding 20 mg/l. Notable high producers included

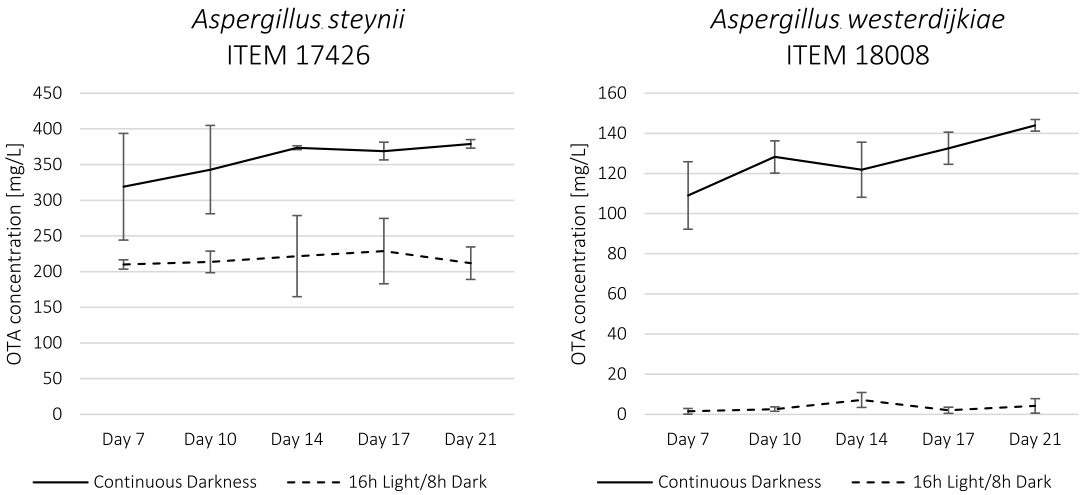


FIGURE 1 Time course of ochratoxin A (OTA) concentrations produced by *Aspergillus steynii* ITEM 17426 and *Aspergillus westerdijkiae* ITEM 18008 under continuous darkness and a 16 h light/8 h dark cycle. Cultures were incubated in CYA medium at 25 °C, and OTA concentrations were measured at five time points. Error bars represent standard deviations from triplicate experiments.

TABLE 4 Ochratoxin A partitioning between aqueous and organic phases during extraction with different organic solvents

| Solvent | OTA amount in stock solution [µg] | OTA amount in aqueous phase [µg] | OTA amount in organic phase [µg] | Observations |
|---|---|---|---|---|
| heptane | 273 ± 17 | 96.5 ± 8.5 | 4.23 ± 0.20 | OTA remained in aqueous phase + precipitation |
| petroleum benzine | 273 ± 17 | 82.8 ± 3.4 | 3.09 ± 0.14 | OTA remained in aqueous phase + precipitation |
| ethyl acetate | 273 ± 17 | 1.30 ± 0.97 | 203 ± 22 | OTA redistributed to organic phase |
| toluene | 206 ± 5 | 6.54 ± 2.30 | 4.71 ± 2.37 | OTA precipitated |
| dichloromethane | 206 ± 5 | 45.4 ± 13.1 | 105 ± 10 | incomplete extraction |
| chloroform | 206 ± 5 | 60.7 ± 10.6 | 99.7 ± 15.7 | incomplete extraction |

*Aspergillus westerdijkiae* ITEM 18008 and *Aspergillus steynii* ITEM 17426, the latter producing the highest OTA concentration of 425 ± 13 mg/l. A previously tested strain, *Aspergillus westerdijkiae* MA 5454, was included as a positive control. In this experiment, it produced OTA concentrations of 158 ± 13 mg/l, consistent with levels observed in earlier media optimisation experiments (151 ± 16 mg/l in CYA medium).

***Purification protocol***

For large-scale OTA production, *Aspergillus steynii* (ITEM 17426) spores were inoculated into 20 ml of CYA medium (500 spores/ml) within magenta boxes. The cultures were incubated under continuous darkness at 25 °C for 14 days. Following OTA extraction and centrifugation, the resulting supernatant was collected and will hereafter be referred to as the stock solution. The stock solution was acidified and six solvents were tested for LLE: heptane, petroleum benzine, ethyl acetate, toluene, dichloromethane, and chloroform. Heptane, petroleum benzine and toluene were not considered as suitable options, as OTA either remained in the aqueous phase or precipitated, sedimenting on the walls of the Eppendorf tubes. In contrast, ethyl acetate, dichloromethane and chloroform demonstrated effective partitioning of OTA into the organic phase and were selected for further evaluation. Ultimately, ethyl acetate was selected as the optimal organic solvent due to its higher recovery efficiency, achieving approximately 75%, compared to only around 50% for chloroform and dichloromethane. Each LLE experiment was performed in triplicate. The results in Table 4 represent the median OTA amount in 1 ml

TABLE 5 OTA partitioning between aqueous and organic phases during back-extraction with different alkaline solutions

| Solutions | OTA amount in ethylacetate solution [μg] | OTA amount in aqueous phase [μg] | OTA amount in organic phase [μg] | Observations |
|---|---|---|---|---|
| 10 g/l $Na_2CO_3$ | 203 ± 22 | 198 ± 23 | 1.90 ± 0.93 | OTA redistributed to aqueous phase |
| 10 g/l $NaHCO_3$ | 203 ± 22 | 185 ± 1 | 2.61 ± 1.38 | OTA redistributed to aqueous phase |
| 5 g/l $Na_2CO_3$ + 5 g/l $NaHCO_3$ | 203 ± 22 | 186 ± 20 | 1.61 ± 0.93 | OTA redistributed to aqueous phase |

in the stock solution, aqueous phase and organic phase after extraction, with standard deviations indicated.

The ethyl acetate phase was collected and another LLE was performed with three different alkaline solutions: 10 g/l $Na_2CO_3$ (pH ~11.5); 10 g/l $NaHCO_3$ (pH ~8.5); a mix of 5 g/l $Na_2CO_3$ and 5 g/l $NaHCO_3$ (pH ~10). Table 5 represents the median OTA amount in 1 ml in the starting ethyl acetate solution, aqueous phase and organic phase after extraction, based on LLE experiments performed in three replications with standard deviations indicated.

All three alkaline solutions showed similar effectiveness in extracting OTA, and as such any of them could be used. For large scale purification a 10 g/l $Na_2CO_3$ solution was used.

The large-scale purification experiments were also performed in triplicate. Two sequential LLE steps were performed. In the first step, using ethyl acetate, 92.0 ± 6.8% of OTA was recovered in the organic phase, while only 0.3 ± 0.2% remained in the aqueous phase. In the second extraction step, using 10 g/l $Na_2CO_3$, OTA was successfully transferred back into the aqueous phase, with 99.4 ± 0.4% recovery and only 0.4 ± 0.2% remaining in the organic phase.

Following LLE, the alkaline aqueous phases from all three extractions were collected and acidified under gentle stirring to remove carbon dioxide, before proceeding with SPE. The OTA concentration was measured in the pass-through fraction after sample loading, as well as in the 50% aqueous methanol wash fraction, to assess retention efficiency. No OTA was detected in either of these fractions, confirming effective adsorption onto the SPE cartridge in all cases. Elution with acidified methanol recovered 91 ± 5% of OTA. A final methanol wash step recovered minor additional amounts of OTA, ensuring near-complete elution.

All collected fractions were pooled, and the volume was reduced from 300 ml to 30 ml using a rotary evaporator before proceeding with preparative HPLC. From the theoretical 224 mg of OTA, 201 mg was recovered, overall resulting in a 90% recovery for this step. A single preparative HPLC run yielded around 6 mg of purified OTA.

***Purity assessment***

The purification process for OTA was evaluated at each step in terms of both recovery efficiency and purity. The cumulative recovery efficiency was calculated as the percentage of OTA retained after each step relative to the initial concentration. Starting from the crude stock solution, OTA accounted for 3 ± 1% of total solids, while OTB represented 1 ± 0%. After extraction with ethyl acetate, recovery efficiency reached 91 ± 0%, and OTA purity improved to 52 ± 1%, with 13 ± 1% OTB remaining. Back-extraction with sodium carbonate solution maintained high OTA recovery (90 ± 6%) and similar purity (51 ± 3%). Subsequent SPE increased OTA purity to 69 ± 1% with a recovery efficiency of 85 ± 7%, though 17 ± 2% OTB was still present. Finally, preparative HPLC achieved the highest OTA purity (100 ± 0.4%) and completely removed OTB and other impurities, with a final recovery efficiency of 76 ± 7%. However, as the OTA reference standard used for calculating OTA concentrations had a certified purity of ≥98%, the calculated value may slightly overestimate the absolute purity, and should be interpreted as analytical standard quality rather than absolute 100%.

Chromatographic analysis further confirmed the improvement in OTA purity throughout the purification process. As shown in Figure 2, panels A and B display the MS/MS and UV chromatograms, respectively, obtained after the SPE step. In both chromatograms, distinct peaks for OTA and OTB are present, along with several smaller peaks indicating co-extracted impurities. Panels C and D show the corresponding chromatograms after preparative HPLC, where only a single, sharp OTA peak is observed. The absence of OTB and additional

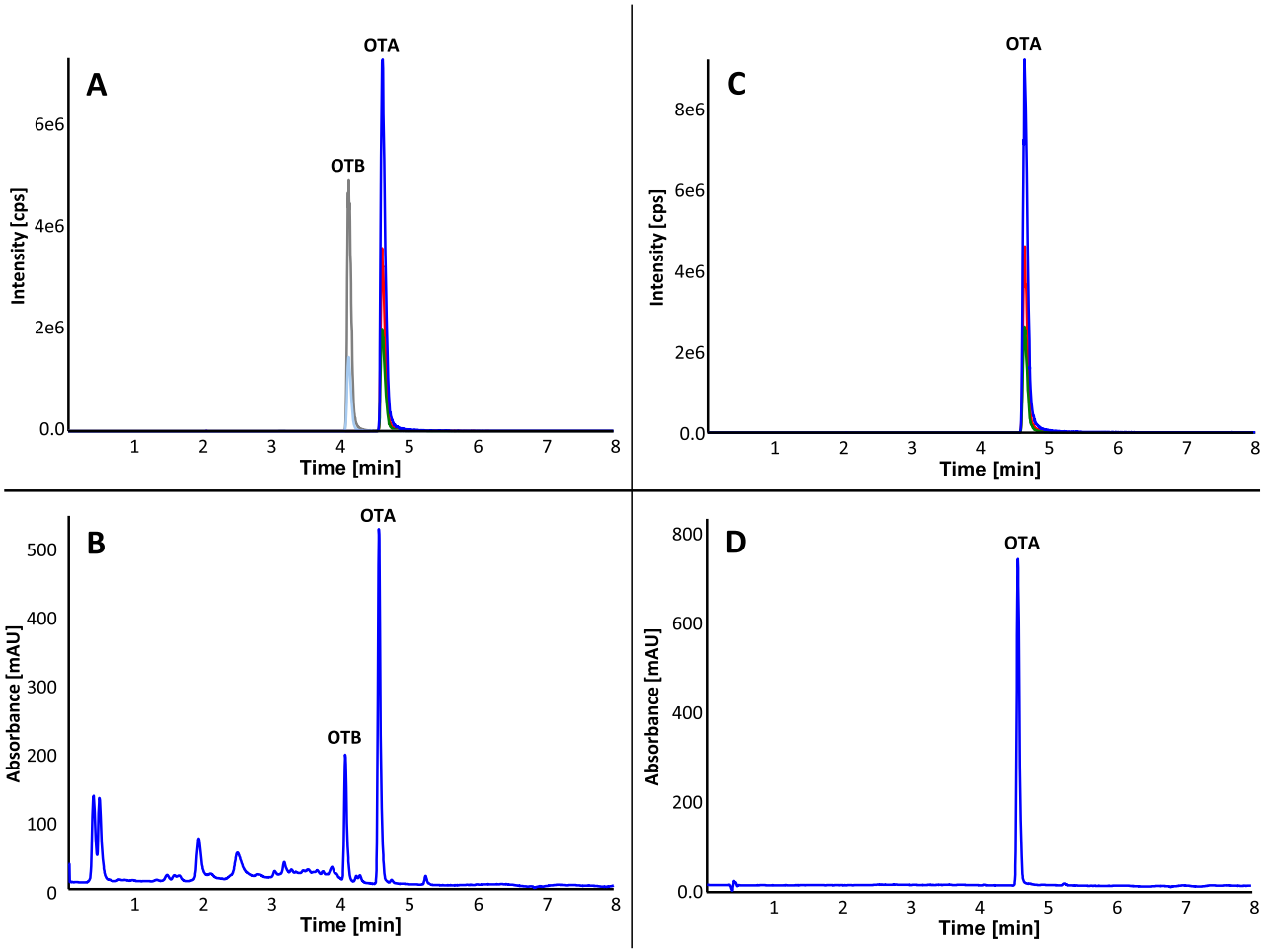


FIGURE 2 MS/MS chromatogram (A) and UV chromatogram at 210 nm (B) of ochratoxin A (OTA) extract after solid-phase extraction. MS/MS chromatogram (C) and UV chromatogram at 210 nm (D) after preparative HPLC.

peaks confirms the effectiveness of preparative HPLC in isolating high-purity OTA.

## 4 Discussion

### *Media optimisation*

In this study, we focused exclusively on OTA production in synthetic and semi-synthetic liquid media, rather than solid, complex cereal-based media. A key reason for this choice was the need for modifiability to support future isotope labelling studies. Complex media containing undefined, high-molecular-weight components such as starch, cellulose, and lignin are unsuitable for this purpose, as these components cannot be fully substituted with isotopically labelled alternatives and may additionally interfere with purification. In contrast, synthetic and semi-synthetic media allow for easier extraction and purification due to their low organic complexity, and they enable the incorporation of labelled carbon sources, a necessary feature for developing OTA production systems compatible with future stable isotope labelling.

The selected media were chosen based on previous studies reporting their ability to support OTA production, with several media yielding concentrations above 20 mg/l (Koteswara Rao *et al.*, 2013; Lillehoj *et al.*, 1978; Wei *et al.*, 1971). One exception was FMM, which was included specifically as a purely synthetic medium to assess the impact of organic components. Among all tested media, CYA consistently yielded the highest OTA concentrations, likely due to its content of yeast extract and trace elements that supply essential nitrogen sources and micronutrients for OTA biosynthesis. While all media contained high sucrose levels, the presence of organic additives appeared to play a critical role. In contrast to FMM, which lacks yeast or malt extract and produced minimal OTA, media such as CYA, FM, and MC supported significantly higher production. BMEAM and YES also yielded lower concentrations, suggesting that specific combinations of organic and min-

eral components are crucial for optimal toxin biosynthesis.

Yields of OTA in liquid cultures vary widely across the literature, depending on strain, medium composition, and cultivation parameters. Many studies using complex or undefined media report low to moderate OTA production. For example, *Penicillium verrucosum* cultured in CYA or YES media had been shown to produce OTA in the tens of mg/l range (Koteswara Rao *et al.*, 2013), and *Aspergillus ochraceus* in YES medium often yields several hundred µg/l (Meca and Ritieni, 2009). In this study, we achieved OTA concentrations of up to 425 mg/l using a liquid semi-synthetic medium, resulting in OTA levels that are among the highest reported for cultures grown under conditions compatible with stable isotope labelling. This demonstrates that it is possible to achieve high OTA yields without relying on complex cereal-based media, establishing a strong foundation for subsequent isotope-labelling work.

***Time and light condition***

The time-course analysis revealed that continuous darkness resulted in significantly higher OTA production than cultivation in a light/dark cycle, with peak production occurring around day 14-17. This observation aligns with research suggesting that light exposure, particularly shorter wavelengths like UV-B and blue light, can inhibit OTA biosynthesis in *Aspergillus ochraceus* and *Aspergillus carbonarius* by disrupting key pathways involved in toxin production (Cheong *et al.*, 2016). Under dark conditions, OTA biosynthetic pathways may remain active and stable, promoting higher OTA concentrations without the interference of light-induced stress responses or degradation mechanisms (Cheong *et al.*, 2016; Zhang *et al.*, 2021). Interestingly, OTA concentrations remained relatively constant after the initial increase around days 7-10, with no significant fluctuations across most time points. This pattern somewhat aligns with previous research, where OTA production typically peaks around 11 days (Bragulat *et al.*, 2001; Lappa *et al.*, 2015; Lillehoj *et al.*, 1978; Valero *et al.*, 2006). However, unlike most studies that report a noticeable drop in OTA concentrations after day 11, our results showed no such decline.

***Strain screening***

The strain screening revealed a wide variability in OTA production, with most *Aspergillus ochraceus* strains and both *Aspergillus westerdijkiae* strains emerging as moderate and high producers, while others, such as *Aspergillus niger*, showed negligible or no OTA production. This disparity may stem from genetic differences among strains that affect OTA biosynthesis pathways. Prior studies have documented similar strain-specific differences, with some studies attributing them to variations in gene clusters responsible for mycotoxin synthesis (El Khoury and Atoui, 2010; Karolewiez *et al.*, 2005). Identifying high-producing strains has practical implications for industrial applications, as these strains could be harnessed for controlled OTA production in research or quality control.

***Purification protocol***

In the LLE step, ethyl acetate was selected over chloroform not only for its higher extraction efficiency in addition to its lower toxicity and greater environmental safety, as chloroform is classified as ozone-depleting and poses disposal challenges, making it a safer and more practical solvent for routine laboratory use. Preparative HPLC was critical for achieving high-purity OTA by effectively separating it from co-extracted impurities, particularly OTB. Due to their structural similarity, OTA and OTB often co-occur in fungal cultures, making their separation challenging. The optimised preparative HPLC method successfully separated these compounds, allowing for the collection of high-purity OTA while also enabling the isolation of OTB, if desired. This dual capability enhances the utility of the method, especially since OTB, though less studied, has some relevance in toxicological research. Access to both purified compounds facilitates future comparative studies on their biological activity and metabolic fate. Earlier protocols often relied on normal-phase silica gel column chromatography as a cost-effective alternative to SPE and HPLC (Scott *et al.*, 1971; Davis *et al.*, 1972). Silica gel can indeed be considered a cheaper alternative to SPE, since it is widely available and provides substantial cleanup. However, it required large volumes of potentially toxic solvents and was particularly labour-intensive, especially when columns had to be manually packed. Reported purities were generally lower than those obtained by preparative HPLC, and separation from structurally related compounds such as OTB was often incomplete. In many cases, recrystallization was required as an additional step to achieve analytical-grade purity, which further increased time and material losses.

The purification protocol proved highly efficient, achieving nearly 100% OTA purity after preparative HPLC with a final recovery efficiency exceeding 75%. This compares favourably to existing methods, which typically report recovery rates of 70-85% (Bunge *et al.*,

1979; Davis *et al.*, 1972; Nesheim, 1969). While recrystallisation can yield purities above 99%, the procedure is technically more demanding, requires additional time, and can result in material loss. In contrast, preparative HPLC as applied here achieved similar purity with greater reproducibility, shorter processing time, and minimal losses. A key advantage of this protocol is its modular design: when high purity is not required, it can be stopped after the LLE step, which already provides approximately 50% purity using affordable solvents and basic equipment. Additional steps like SPE and preparative HPLC can be added in case of higher purity needs, allowing the method to be adapted based on resources and goals. Moreover, by avoiding toxic solvents like chloroform and reaching OTA yields of around 400 mg/l, which is substantially higher than earlier protocols (approximately 46 mg/l) (Davis *et al.*, 1972), the method demonstrates both safety and scalability.

This study presents an optimised workflow for OTA production and purification in liquid media, as well as a robust foundation for subsequent work focused on producing uniformly labelled OTA using labelled carbon sources. The protocol yields OTA of analytical standard purity, enabling its use not only in research but also as a reference standard for analytics.

## Supplementary materials

Data is available on https://doi.org/10.1163/18750796-bja10029 under Supplementary Materials.

**Table S1.** List of fungal strains used in OTA screening, with measured OTA concentrations.

**Table S2.** OTA concentrations in varying volumes of CYA medium after two weeks incubated with *Aspergillus steynii* ITEM 17426.

**Table S3.** Effect of rinsing on OTA extraction efficiency and reproducibility.

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## Authors' contribution

F.P.: Writing – original draft, data curation, formal analysis, investigation, visualization, conceptualization. G.W.: writing – reviewing and editing, project administration, supervision, conceptualization. G.A.: writing – reviewing and editing, resources. A.S.: writing – reviewing and editing, resources. F.B.: writing – reviewing and editing, conceptualization, funding acquisition, methodology, project administration, supervision. All authors have read and agreed to the published version of the manuscript.

## Conflict of interest

The authors declare no conflict of interest. F.B. is editor in chief of World Mycotoxin Journal. He has not been involved in the review and the decision process of this paper.

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