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Combination of Ultrasonic-assisted Aqueous Two-phase Extraction with Solidifying Organic Drop-dispersive Liquid–liquid Microextraction for Simultaneous Determination of Nine Mycotoxins in Medicinal and Edible Foods by HPLC with In-series DAD and FLD

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Abstract

A novel method based on ultrasonic-assisted aqueous two-phase extraction (UAATPE) coupled with solidifying organic drop-dispersible liquid–liquid microextraction (SOD-DLLME) was developed for simultaneous determination of nine mycotoxins (aflatoxins of B1, B2, G1, G2, and M1, ochratoxin A, zearalenone, deoxynivalenol and patulin) in medicinal and edible foods by high-performance liquid chromatography (HPLC) with diode array detector (DAD) and fluorescence detector (FLD) in series. Using an aqueous two-phase system (ATPS) of acetonitrile and $(NH_4)_2SO_4$ as the extractant, the effects of the ATPS composition, extraction temperature and time were investigated respectively to extract the mycotoxins from the samples. Also, SOD-DLLME conditions including type and volume of extractant and dispersant were optimized by the single factor experiments. The optimum conditions were as follows: the ATPS composition of 34.0% acetonitrile concentration (w/w) and 22.0% $(NH_4)_2SO_4$ concentration (w/w), pH 6.0, extraction temperature 40 °C, ultrasonic time 10 min for UAATPE; 1-dodecanol 600 μL as extractant, acetonitrile 1.0 mL as dispersant, and vortex-assisted time 1.0 min for SOD-DLLME. By means of HPLC–DAD-FLD detection, nine mycotoxins had good linearity in the range of 0.5 – 200.0 ng/mL ($R^2 \geq 0.9991$). LODs and LOQs were in the range of 0.01563 – 0.5161 ng/mL and 0.05210 – 1.720 ng/mL, respectively. The average recoveries and intra-day and inter-day precisions were 82.77 – 103.2%, 1.1 – 3.4% and 1.5 – 4.3%, respectively. The proposed method was successfully applied to simultaneous determination of multiple mycotoxins in black bean, black sesame, lotus seed, apricot kernel and litchi, demonstrating the presence of five different mycotoxins in these samples.

Keywords Ultrasonic-assisted aqueous two-phase extraction · Dispersed liquid–liquid microextraction · High-performance liquid chromatography · In-series DAD-FLD detection · Mycotoxins · Medicinal and edible foods

Introduction

Medicinal and edible foods such as fruits, seeds, pulp and/or peel/pericarp from dietary plants and traditional medicinal herbs play an important role in human nutrition and health because of their nutritional properties and bioactive principles (Huang et al. 2010; Shi and Jiang 2020; Que et al. 2017a. 2017b). Black beans, black sesame, lotus seeds and apricot kernel as the archetypal medicinal and edible plants are widely applied in China (Chinese Pharmacopoeia Commission 2020a). Litchi produced in South China possesses a sweet odor of rose, delicious taste, and good nutritional value, its dried fruit exhibited multiple bioactivities such as hypoglycemic, anticancer, antibacterial, anti-hyperlipidemic,

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anti-platelet, and antiviral (Ibrahim and Mohamed 2015). Taking into account the important medicinal and edible values, these foods are favored by consumers in south China, and prepare delicious foods with various local flavors and health functions. However, owing to unsanitary conditions during planting, harvesting, processing, storage and transportation, they will be polluted to different degrees. Especially seeds and fruits are susceptible to mycotoxins contamination caused by various fungi with moldy growth and spoilage in a tropical climate (Paterson and Lima 2010; Yang et al. 2020). After ingestion, mycotoxins will cause several diseases in humans and animals (Tripathy et al. 2014; Thanushree et al. 2019). Therefore, mycotoxins contamination is very harmful to human and animal, and persistent efforts have been devoted to controlling and monitoring mycotoxins. So far, more than 400 mycotoxins have been identified from a variety of foods and agricultural commodities worldwide (Yang et al. 2020). Among them, aflatoxins (AFs), ochratoxin A (OTA), deoxynivalenol (DON), patulin (PAT), zearalenone (ZEN) are quite common contaminants occurring jointly in a wide range of foods (the chemical structures are presented in Fig. S1) (Yang et al. 2020). Due to high toxicity of these compounds, many countries and organizations have formulated the maximum limits (MLs) in foodstuffs, feeds, and other matrices intended for human or animal consumption (European Commission 2006; National Standard of the People's Republic of China National 2017; Chinese Pharmacopoeia Commission 2020b). In recent years, monitoring the mycotoxins in medicinal herbs has been paying more and more attention (Wang et al. 2020; Liu et al. 2016, 2019, 2018; Sun et al. 2018; Cho et al. 2019). Especially in South China, the foregoing medicinal and edible foods as major ingredients are very prone to occurrence of mycotoxins contamination in the moist climate. Moreover, multiple mycotoxins may be found in the same product because a single species of fungi can produce several toxic metabolites, even several species of fungi can be simultaneously present and produce different toxins (Tripathy et al. 2014; Thanushree et al. 2019; Yang et al. 2020). Up to now, there have been a few related reports about monitoring the mycotoxins in these medicinal and edible foods.

In order to control the mycotoxins in foodstuffs, a reliable analytical method is essential to obtain accurate data for establishment of the regulatory limits and evaluation of the infection risk. In the last few years, strenuous efforts have been devoted to develop analytical methodologies for the effective determination of mycotoxins, particularly multi-mycotoxins methods (Yang et al. 2020; Cho et al. 2019; Liu et al., 2019, 2018; Rahmani et al. 2009). Among them, high-performance liquid chromatography-tandem mass spectrometry (HPLC–MS/MS) and HPLC with FLD or DAD are preferred methods of mycotoxins analysis (Yang et al. 2020; Chinese Pharmacopoeia Commission 2020b; Rahmani et al. 2009; General Administration of Quality Supervision 2016). However, sample pretreatment is fundamental and indispensable steps for accurate and sensitive determination of trace mycotoxins in complex sample matrices (Yang et al. 2020; Chinese Pharmacopoeia Commission 2020b; Sun et al. 2018; Cho et al. 2019; Liu et al. 2019; Liu et al. 2018; Rahmani et al. 2009; General Administration of Quality Supervision 2016). Usually, extraction of mycotoxins from solid samples is the first step in sample preparation, followed by clean-up procedures to improve the sensitivity and specificity of detection methods (Chinese Pharmacopoeia Commission 2020b; Liu et al. 2019, 2018; Rahmani et al. 2009; General Administration of Quality Supervision 2016). Most of protocols use acetonitrile/methanol or acetonitrile–water/methanol–water mixture as a solvent to extract mycotoxins from samples. In addition to filtration and centrifugation, further clean-up steps are needed to improve sensitivity and selectivity (Yang et al. 2020; Chinese Pharmacopoeia Commission 2020b; Liu et al. 2019; Liu et al. 2018; Rahmani et al. 2009; General Administration of Quality Supervision 2016). The different sample pretreatment techniques have been developed to match a solvent extraction step for purification and enrichment of trace mycotoxins in various sample matrices such as solid-phase extraction (SPE) (Dong et al. 2019), dispersive solid-phase extraction (DSPE) (Wang et al. 2020), liquid–liquid extraction (LLE) (Liu et al. 2016; Romera et al. 2018), solid-phase microextraction (SPME) (Andrade and Lancas 2017), QuEChERS (quick, easy, cheap, effective, rugged and safe) (Liu et al. 2018; Colli et al. 2020), immunoaffinity column (IAC) (Liu et al. 2019, 2018; Rahmani et al. 2009), magnetic solid phase extraction (MSPE) (Zhao et al. 2020), and dispersive liquid–liquid micro-extraction (DLLME) (Yang et al. 2020), have been developed to match a solvent extraction step for purification and enrichment of trace mycotoxins in diverse sample matrices. With the function of biphasic extraction, aqueous two-phase extraction (ATPE) can achieve separation of the different components in two phases based on the difference of surface properties, charge action and various forces such as van der Waals force, hydrophobic bond, hydrogen bond and ionic bond (Assis et al. 2020; Iqbal et al. 2016). Compared to single solvent extraction, an ATPS can selectively extract target analytes into the top or bottom phase, reducing the interference of sample matrix. An ATPS composed of two phase-components (e.g. polymer–polymer, polymer-salt, and alcohol-salt, etc.) has an option to adjust the extraction performance by controlling the composition of the phase-forming components, expanding a wide range of extractants (Mai et al. 2020; Zhou et al. 2018). Moreover, water-rich phases would boost the penetration of the extractants in the food matrix. An acidic solution can break the strong bonds between the analytes and other food components such as protein and sugars, thus enhancing the extraction efficiency

(Yang et al. 2020; Rahmani et al. 2009). For example, the green ATPSs formed by polyethylene glycol and salt as alternatives to organic solvents and/or toxic reagents have exhibited high extraction capacity to mycotoxins, keeping a good connection with the subsequent clean-up procedures (Soares et al. 2014, 2017). DLLME is superior to others in respect of simple configuration, rapid operation, lower costs and high enrichment efficiency. It is frequently combined with clean-up techniques such as microwave-assisted extraction (MAE), QuEChERS and DSPE, etc. (Sajid and Alhooshani 2018; Wang et al. 2018; Trevisan et al. 2017; Farajzadeh et al. 2019). For mycotoxins or other analytes in solid samples, most cases are usually subjected to single-phase solvent extraction with acetonitrile or acetonitrile–water mixture prior to DLLME procedures, inevitably resulting in more complex process (Rausch et al. 2020; Yazdanfaret al. 2019). Therefore, novel or modified methods are needed to simplify extra steps or reduce unnecessary errors. ATPE combining with DLLME is a resolution to avoid this situation because of integrating extraction with purification and enrichment in a one-step procedure.

Although high-sensitive HPLC–MS/MS has been used for determination of multiple mycotoxins in different species of functional and medicinal herbs, measurement recoveries and uncertainty had a wide fluctuation due to matrix effect (Sun et al. 2018; Cho et al. 2019; Liu et al. 2019, 2018; Rahmani et al. 2009). Compared with HPLC–MS/MS, which is not easily accessible for most laboratories, HPLC is simple with low cost, and easy to operate. In this work, a method of combining APTE with DLLME was developed for simultaneous extraction and enrichment of the mycotoxins in black beans, black sesame, lotus seeds, apricot kernel and litchi by HPLC coupled to series connection of DAD and FLD, so that the mycotoxins in a wide range of the sample matrices can be quantitatively determined to evaluate contamination levels. To this end, an ATPS of acetonitrile/$(NH_4)_2SO_4$ was used for UAATPE, and followed by screening of a modified DLLME system to connect with UAATPE procedure for simultaneous extraction and enrichment of the mycotoxins in the samples. Accordingly, UAATPE conditions including the composition of the ATPS, pH, extraction temperature and time were investigated to enhance extraction of the mycotoxins. In the DLLME process, key factors such as extractant and extractant volume, dispersant and dispersant volume, vortex-assisted time were investigated to maximize extraction recoveries of the mycotoxins. Based on the UV and fluorescence characteristics of the mycotoxins before and after derivatization, HPLC conditions were optimized through in-series DAD and FLD detection. Finally, UAAPTE-DLLME coupled with HPLC–DAD-FLD would be applied to simultaneous determination of the mycotoxins in medicinal and edible foods (See Fig. 1).

Materials and Methods

Chemicals and Materials

Methanol, acetonitrile of HPLC grade were obtained from Merck Ltd. (Germany). Ethanol, n-hexane, ammonium sulfate and n-hexane were purchased from Zhiyuan Chemical Reagent Co., Ltd. (Tianjin, China). Chloroform, carbon tetrachloride, dimethyl carbonate, dimethyl carbonate, methyl salicylate, ethyl salicylate, 1-dodecanol and concentrated hydrochloric acid were obtained from Guangzhou Chemical Reagent Factory (Guangzhou, China). Trifluoroacetic acid (TFA) was purchased from Maclean Biochemical Technology Co., Ltd. (Shanghai, China). The standards of aflatoxins (AFs including AFG1, AFG2, AFB1, AFB2, AFM1), ochratoxin A (OTA), zearalenone (ZEN), deoxynivalenol (DON) and patulin (PAT) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

At least 500 g of dried samples of black beans, black sesame seeds, lotus seeds and apricot kernel were respectively purchased from Qingping Market in Guangzhou, between March 2017 and April 2018. A year-old dried litchi was collected from farmers in Dongguan, and the pulp was used as the sample to be tested after the pericarp and the core were removed. All samples were pulverized, sealed in plastic packages, and then stored in desiccators at room temperature. Analysis of samples was completed in 30 days.

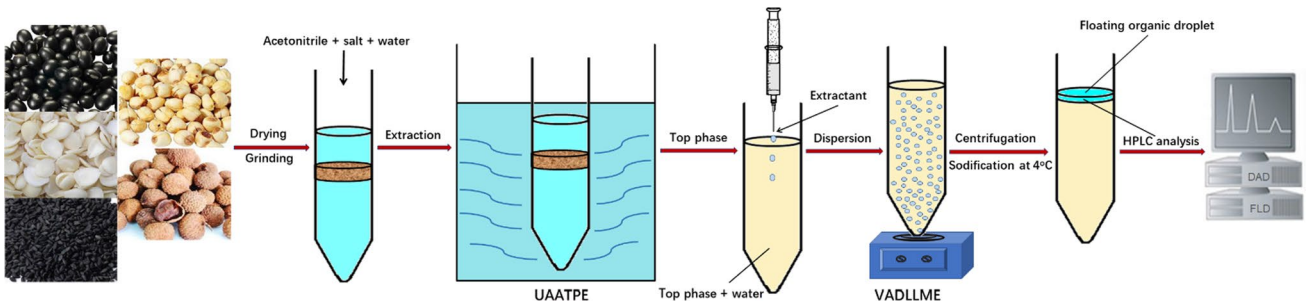

Fig. 1 The process of UAATPE-DLLME coupled to HPLC–DAD-FLD for analysis of the mycotoxins in medicinal and edible foods

Instrumentations

HPLC analysis of the mycotoxins was performed on a Shimadzu LC-20AD chromatograph equipped with RF-20A FLD detector and SPD-20A DAD detector (Shimazu Co., Ltd., Japan); LabSolution Version 5.92 software was used for calculation and quantification (Shimazu Co., Ltd., Japan). UAATPE was carried out on a KQ-2200B ultrasonic device with frequency and temperature controller (Kunshan Ultrosonic Instrument Co., Ltd., China); DLLME extraction was carried out on a HD-2500 multi-tube vortex mixer (Hangzhou Youning Instrument Co., Ltd., China); After the vortex, centrifugation of the resulting mixture was performed in a TD6-Benchtop centrifuge (Hunan Pingfan Science and Technology Co., Ltd., China), respectively; The mycotoxins were derived in the DK-98-II thermostatic water bath (Tianjin Taisite Instrument Co., Ltd., China); The top phase collected from UAATPE was blown to dryness under a gentle stream of nitrogen gas by ZGDCY-12 Termovap sample concentrator (Shanghai Zigui Instrument Co., Ltd., China).

Preparation of Standard Solutions

0.1 mg/mL of the stock standard solutions of AFs (AFG_1, AFG_2, AFB_1, AFB_2, AFM_1), OTA, ZEN, DON and PAT was prepared in acetonitrile. The stock solutions were stored at 4 °C before use. According to the requirements of the experiment, 0.1 – 10 μg/mL of working standard solutions were freshly prepared by diluting and mixing each stock solution with acetonitrile step by step.

UAATPE and SOD-DLLME procedure

1.0 g of sample powder or 0.1 mL of mixed standard solution of mycotoxins was taken into a glass tube, then 1.256 g $(NH_4)_2SO_4$ and 2.51 mL water was added, respectively. After dissolving, 2.49 mL acetonitrile was added to form an ATPS, and the mixture was placed in the ultrasonic bath for extraction of mycotoxins for 10 min at 40 °C and 40 kHz with the output power of 200 W. Then, allowed it to stand until two phases be separated. 1.0 mL of the top phase, 600 μL of 1-dodecanol and 4 mL of water were added into a centrifuge tube in turn. The mixture was vigorously vortexed for 1 min to allow the complete extraction. After being centrifuged at 2000 rpm for 5 min, the mixture was placed in a refrigerator for solidification of the top phase at 4 °C for about 10 min. Afterward, the bottom phase was removed by a syringe with needle, the solidified top phase was kept at room temperature until thawed for the following derivatization and HPLC analysis.

Derivatization of Mycotoxins

100 μL of TFA was added to the above extract liquefied. The mixture was subjected to derivatization of mycotoxins in a water bath at 40 °C for 20 min, and then allowed to separate into layers. The top layer was collected and passed through 0.22 μm membrane for HPLC analysis.

HPLC Analysis with in-series DAD and FLD

The quantification of the mycotoxins in the sample solution was conducted by HPLC coupled to in-series FLD and DAD. Using the mixture of 0.5% acetic acid–water (v/v, A) and 50% methanol–acetonitrile (v/v, B) as the mobile phase, the mycotoxins in the above sample solution was chromatographically separated on a Phenomenex Gemini C18 column (250 mm × 4.6 mm, 5 μm) according to the following gradient elution: 0–7 min, 25% B; 7–15 min, 25–70% B; 15–25 min, 70% B; 25–35 min, 70–100% B. Injection volume, flow rate and column temperature were 10 μL, 1.0 mL/min, and 40 °C, respectively. Detection was performed with DAD and FLD detectors, connected in series, and the FLD detection procedure was as followed: 0–20 min, excitation wavelength of 360 nm and emission wavelength of 430 nm; 20–25 min, excitation wavelength of 275 nm and emission wavelength of 440 nm; 25–35 min, excitation wavelength of 333 nm and emission wavelength of 460 nm.

Results and discussion

Optimization of UAATPE conditions

Screening of an ATPS

The ATPS made by a short-chain solvent and a salt, offers the advantages of low viscosity, easy demixing, convenient to subsequent processing and solvent recycling, so ethanol with nontoxicity and acetonitrile with chromatographic use were used as phase-forming components to construct an ATPS with $(NH_4)_2SO_4$. According to the phase formation diagrams (Zhang et al. 2015), the ATPSs of ethanol/$(NH_4)_2SO_4$ and acetonitrile/$(NH_4)_2SO_4$ were prepared, respectively, for screening of an appropriate biphasic extraction system. Thus, the effects of two ATPSs on extraction of the mycotoxins analyzed were investigated under the same conditions of the ATPS composition and the phase ratio.

Both two ATPSs were spontaneously formed while mass composition at 17.0% (w/w) of $(NH_4)_2SO_4$ and 24.0% (w/w) of acetonitrile or ethanol, but nine mycotoxins exhibited diverse extraction performances in two ATPSs. The results demonstrated that nine mycotoxins preferred to be extracted into

the rich-acetonitrile/ethanol top phase, whereas some matrix components with great polarity were left in the bottom phase. As a result, the interferences from the sample matrix could be eliminated or partially removed. From Fig. 2a, acetonitrile/$(NH_4)_2SO_4$ system obviously had higher recoveries than that of the ATPS made by ethanol and $(NH_4)_2SO_4$. As shown in Fig. 2b, the recoveries of nine mycotoxins were in the range of 71.59−87.61% with acetonitrile/$(NH_4)_2SO_4$ system as the extractant while at 1:1 of the phase ratio (e.g. the volume ratio of the top phase to bottom phase). However, the recoveries of all mycotoxins for ethanol/$(NH_4)_2SO_4$ system were lower than 55.16%. Therefore, acetonitrile/$(NH_4)_2SO_4$ system as biphasic extractant was suitable for extraction of nine mycotoxins.

Effect of composition of the ATPS

The biphasic extraction property of an ATPS to the mycotoxins depends on the ATPS composition of phase-forming components, which mean to dictate the polarity and the volume of the top or bottom phase (Zhang et al. 2015). Accordingly, the effect of the composition of the ATPS on extraction of the mycotoxins was investigated by controlling the amounts of acetonitrile and $(NH_4)_2SO_4$. According to the phase diagram, there is a wider range of formation of an ATPS while at 15.0% of $(NH_4)_2SO_4$ concentration (w/w). Thus, the influence of acetonitrile concentration on extraction of the mycotoxins was firstly investigated, followed by investigation of $(NH_4)_2SO_4$ concentration.

As shown in Fig. 3a, the recoveries of the mycotoxins increased with the increase of acetonitrile concentration, but decreased at higher than 34.0% of acetonitrile concentration (w/w). Subsequently, $(NH_4)_2SO_4$ concentration was further investigated when kept at 34.0% of acetonitrile concentration (w/w). From Fig. 3b, the recoveries of the mycotoxins increased with the increase of $(NH_4)_2SO_4$ concentration, and almost reached maximum values at 22.0% $(NH_4)_2SO_4$ concentration (w/w). The above results demonstrated that the ATPS composition should be controlled at 34.0% of acetonitrile concentration (w/w) and 22.0% of $(NH_4)_2SO_4$ concentration (w/w) to transfer the mycotoxins to the top phase rather than the bottom phase.

Effect of pH

Since the mycotoxins are susceptible to acidity, different pH will affect their species or forms, resulting in change of partition coefficient in an UAATPE process even decomposition. The effect of pH on extraction of mycotoxins was investigated in the range of pH 3.0 − 7.0. From Fig. 3c, the recoveries of the mycotoxins increased gradually with the increase of pH, and reached the highest values at pH 6.0 except for OTA and PAT. Under the condition of strong acid, some mycotoxins were slightly decomposed, the other's mycotoxins may lead to the protonation. As a result, the partition coefficient of the mycotoxins became smaller in the top phase with relatively small polarity, and their extraction recoveries decreased. Comprehensively, all mycotoxins could achieve higher recoveries, thus pH 6.0 was selected for the following experiments.

Effect of temperature and time

Extraction temperature and time are key factors that influence extraction of target analytes. Appropriate temperature and time can provide enough energy to improve extraction recoveries of the mycotoxins. Also, the effects of extraction temperature and time on the recoveries of the mycotoxins were investigated in the range of 20 − 60 °C and 5 − 25 min, respectively. As shown in Fig. 3d, the recoveries of the mycotoxins increased with the increase of extraction

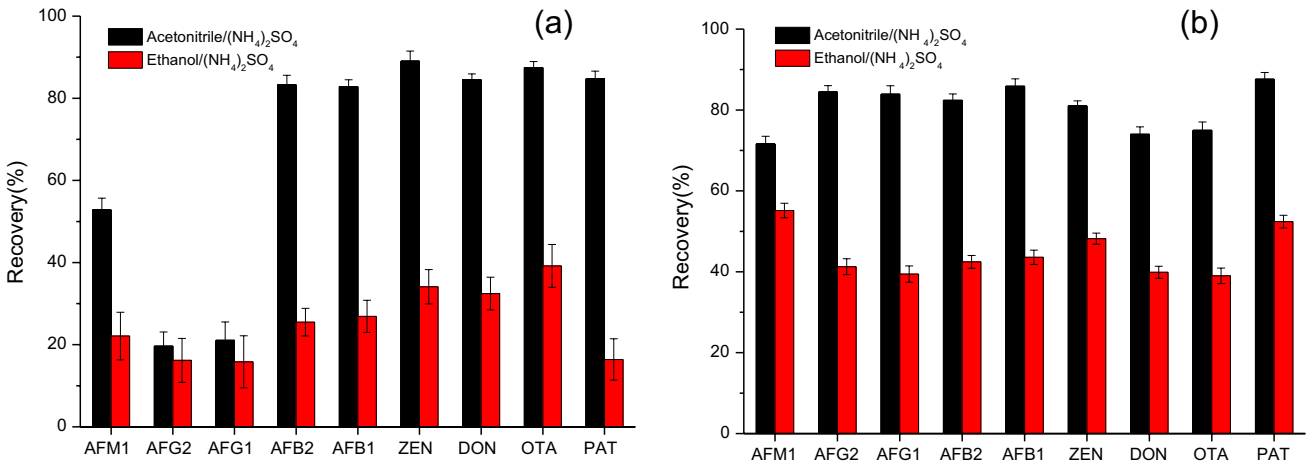

Fig. 2 The effects of two ATPSs at the same composition [17.0% $(NH_4)_2SO_4$ concentration (w/w) and 24.0% acetonitrile/ethanol concentration (w/w)] (**a**) and the phase ratio at 1:1 [20.0% $(NH_4)_2SO_4$ concentration (w/w) and 37.0% acetonitrile, and 18.0% $(NH_4)_2SO_4$ concentration (w/w), 25.0% ethanol concentration] (**b**) on the recoveries of the mycotoxins

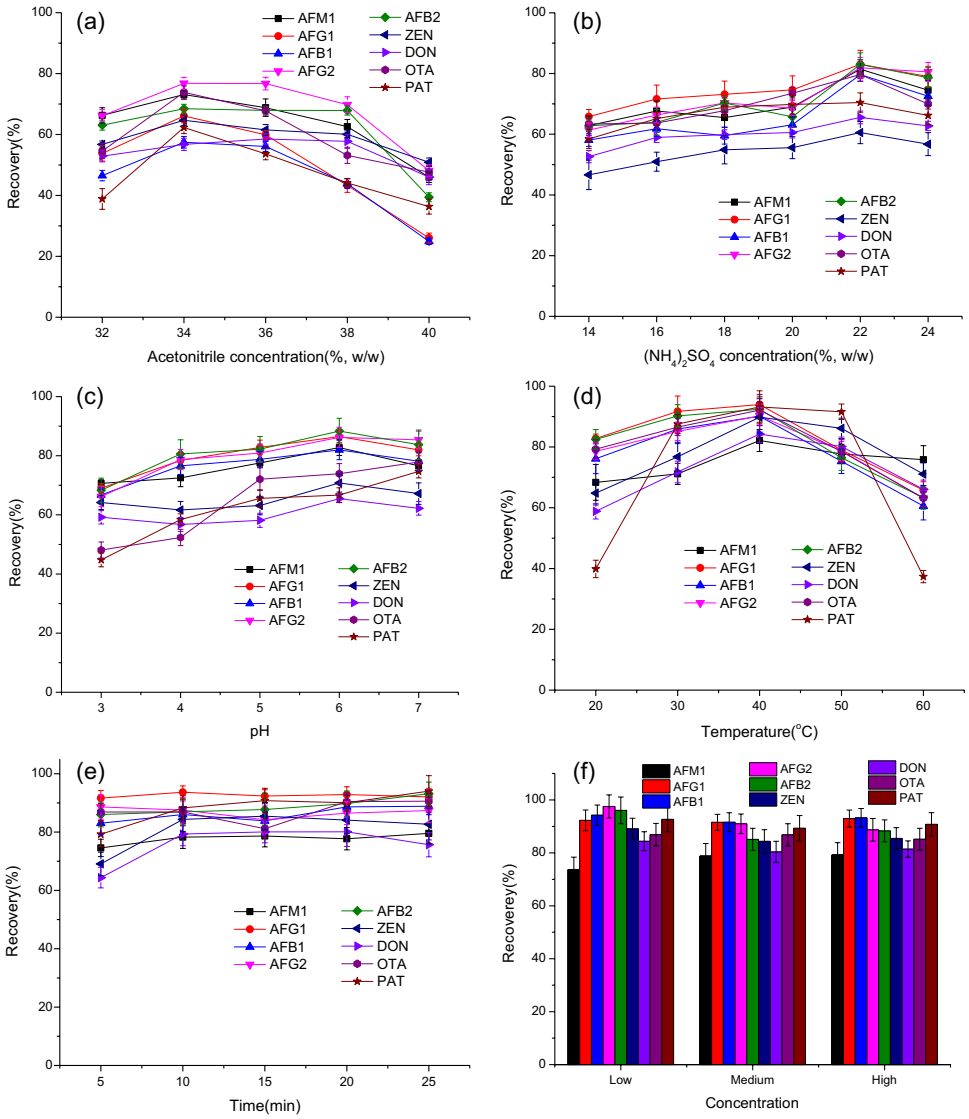

Fig. 3 The effects of acetonitrile concentration (**a**), $(NH_4)_2SO_4$ concentration (**b**), pH (**c**), extraction temperature (**d**), extraction time (**e**) and different concentration (**f**) on the recoveries of the mycotoxins

temperature, and then decreased at more than 40 °C. From Fig. 3e, the impaction of extraction time on the recoveries was relatively smooth and steady, taking 10 min of extraction time for the mycotoxins was enough to reach higher recoveries. Therefore, extraction temperature and time were set at 40 °C and 10 min as the best option for extraction of the mycotoxins.

Effect of mycotoxins concentration

In addition to the extraction performance of the ATPS, the limited ATPS volume influences the extraction capacity of the mycotoxins. In order to link to the subsequent DLLME process, the effects of different concentrations of the mycotoxins on extraction of the mycotoxins were investigated at three levels according to the above conditions optimized. The three levels of the mixed standard solutions consisted of low, medium and high concentrations of the mycotoxins from 25.0 to 500 ng/mL according to detection sensitivity of each mycotoxin. From Fig. 3f, the recoveries of the mycotoxins at three levels ranged between 73.71 and 97.54%. In the results as a whole, the low concentration achieved slightly higher recoveries than that of medium and high concentration, but there was no significant difference while using 5 mL of the ATPS. The results indicated that the extraction capacity of the ATPS could recover the mycotoxins in the above concentration range.

Finally, the optimized extraction conditions for the mycotoxins were summarized as follow: the ATPS composition of 34.0% acetonitrile concentration (w/w) and 22.0% of $(NH_4)_2SO_4$ concentration (w/w), pH 6.0, 40 °C of extraction temperature, 10 min of ultrasonic time. With 5 mL of the ATPS as the extractant, nine mycotoxins ranged in the concentration from 25.0 ng/mL to 500 ng/mL could be extracted to the top phase with higher recoveries.

Optimization of DLLME conditions

Effect of extractants

In the DLLME process, selection of an appropriate extractant is key to preconcentrate the target analytes. Based on the physiochemical properties of the mycotoxins, chloroform, carbon tetrachloride, dimethyl carbonate, methyl salicylate, ethyl salicylate and 1-dodecanol were used as the extractant to extract these mycotoxins (Sajid and Alhooshani 2018). The two formers are often used as DLLME extractant, the latter four solvents with nontoxicity are attempted to serve the mycotoxins. As shown in Fig. 4a, chloroform, methyl salicylate, ethyl salicylate, and 1-dodecanol exhibited the higher recoveries to the mycotoxins, and illustrated different phase-separation behaviors (See Fig. S2). Relatively, methyl salicylate, ethyl salicylate, and 1-dodecanol were less toxic than chloroform. However, the viscosity of the salicylates located to the bottom phase of the ATPS was too high for separation and operation, and the chromatographic peaks would make serious interference to detection of DON. Considering the lower density and higher melting point, 1-dodecanol located at the top phase could be solidified at low temperature or in ice water, which was also more to be conveniently collected by solidification of floating organic drop and two-phase separation before and after derivatization. Thus, 1-dodecanol could be selected as the solidifying extractant for modification of the common DLLME process [named solidifying organic drop solidifying organic drop-dispersible liquid–liquid microextraction (SOD-DLLME)] in next experiments.

Effect of extractant volume

In order to ensure full enrichment of target analytes, the effect of 1-dodecanol volume was further investigated in range of 400 – 900 μL. From the results in Fig. 4b, the recoveries of the mycotoxins were improved with increase of 1-dodecanol, and then reached relatively stable values. The results illustrated that 600 μL of 1-dodecanol could be sufficient to extract nine mycotoxins. Thus, 600 μL of 1-dodecanol was chosen for the following experiments.

Effect of dispersants

In the SOD-DLLME process, an appropriate dispersant can homogenize two immiscible solutions for increase of the contact area between the extractant and the aqueous sample, resulting in target analytes that are extracted from the sample to finely homogeneous droplets. To disperse the extractant to the aqueous sample, methanol, ethanol, acetonitrile, and acetone were screened for dispersion of 1-dodecanol. The results in Fig. 4c demonstrated that acetonitrile could have achieved higher recoveries for the mycotoxins. Moreover, acetonitrile as both the suitable dispersant and

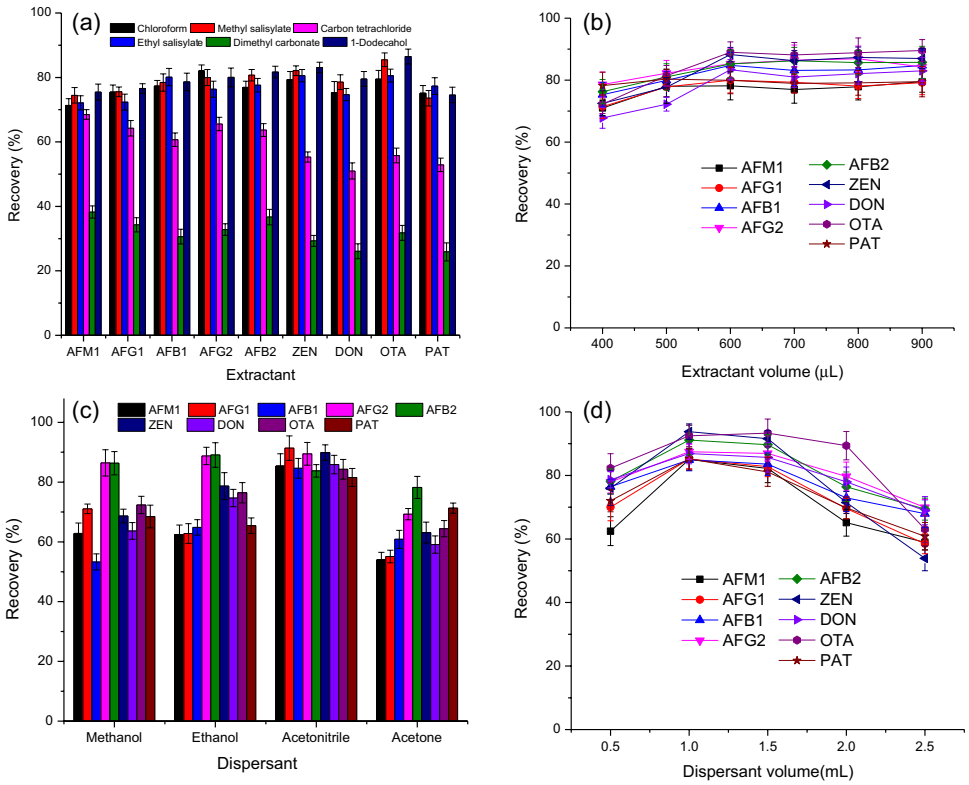

Fig. 4 The effects of extractant (**a**), extractant volume (**b**), dispersant (**c**) and dispersant volume (**d**) on the recoveries of the mycotoxins

the phase-forming component could directly connect to the above UAATPE process without being blown to dryness (Chen et al. 2020). Therefore, acetonitrile was selected as the dispersant in the following experiment.

Effect of dispersant volume

To effectively disperse the extractant to the aqueous sample, the moderate volume of the dispersant must be adopted for complete dispersion so as to ensure high extraction capacity, because the dispersant volume is too small or large to attain higher recoveries for target analytes due to incomplete dispersion or dissolution loss of the extractant. Due to the rich-acetonitrile, the top phase from UAATPE as the dispersant can be directly linked to SOD-DLLME procedure to avoid extra sample preparation steps such as evaporation or concentration and re-dissolution, etc. Accordingly, the effect of the top phase volume on the recoveries of the mycotoxins was investigated while 1-dodecanol was kept at 600 µL. As shown in Fig. 4d, the recoveries of the mycotoxins increased with increase of the dispersant volume, and illustrated the maximum value at 1.0 mL of the rich-acetonitrile top phase. Consequently, 1.0 mL of the top phase solution was selected as the dispersant for SOD-DLLME enrichment of the mycotoxins.

In addition, the effects of vortex-assisted time and NaCl added on the recoveries of the mycotoxins were also investigated. The results showed that the recoveries of the mycotoxins had no significant change between 1 and 5 min without addition of NaCl. This might be because the top phase had been saturated by $(NH_4)_2SO_4$. In summary, SOD-DLLME optimum conditions were 600 µL of 1-dodecanol as the extractant, 1.0 mL of the rich-acetonitrile top phase as the dispersant solvents, 1 min of vortex-assisted time, respectively.

Method performance

HPLC separation and DAD-FLD detection

The mixed standard solution and the above extract were subjected to TFA derivatization, and the top layer was used for HPLC analysis with DAD and FLD in series. As shown in Fig. 4, nine common mycotoxins were well separated within 35 min with the mixture of 50% methanol–acetonitrile (v/v, A) and 0.5% acetic acid–water (v/v, B) as mobile phase in gradient elution, the resolution (Rs) between any two peaks was more than 1.5, and the theoretical plates were more than 3000. Based on UV absorption spectra, PAT, AFM1, DON, ZEN, and OTA could be detected by DAD at 277, 360, 218, 276, and 334 nm, respectively [See Fig. 5a]. Under the excitation of the optimized wavelengths, fluorescence intensity of AFM1, ZEN, OTA, AFG1, AFB1, AFG2, and AFB2 could be detected by FLD through TFA derivatization [See Fig. 5b], and AFM1, ZEN and OTA exhibited higher sensitivity than DAD detection. Therefore, using HPLC coupled to in-series connection of DAD and FLD improved high-throughput detection of the mycotoxins, and could meet the requirements of simultaneous determination of nine mycotoxins in real samples.

Linearity, LOD and LOQ

To evaluate quantification performance of the proposed method, a series of different concentrations of the mixed standard solutions were prepared by diluting the

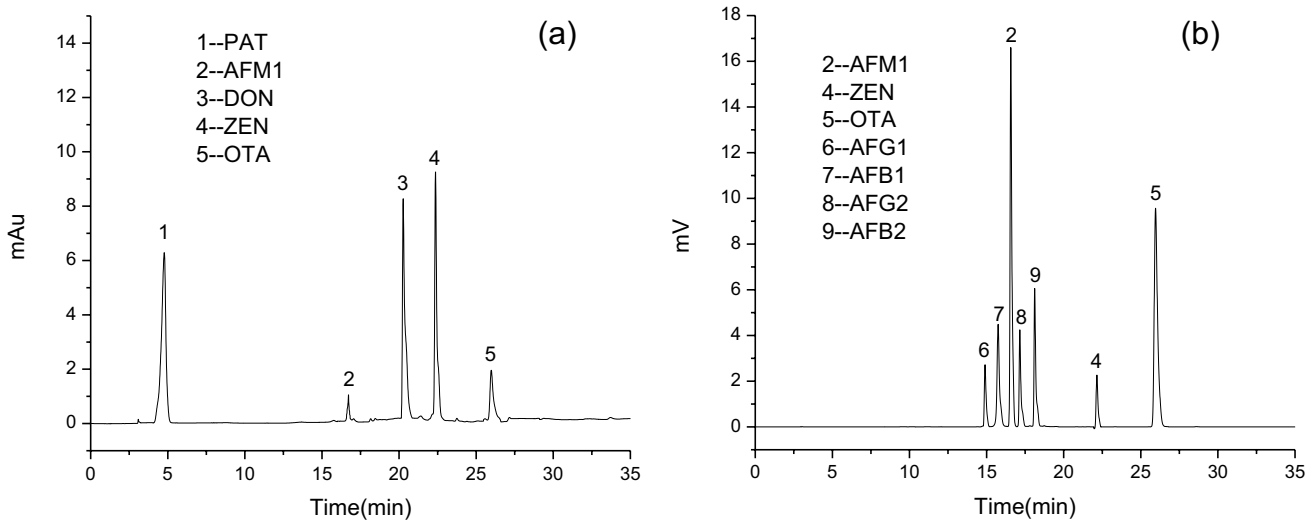

Fig. 5 HPLC chromatograms of mixed standard solution of the mycotoxins by DAD detection (**a**) and FLD detection (**b**): 50 ng/mLof AFB1, AFB2, AFG1, AFG2 and 125 ng/mL of PAT, AFM1, DON, ZEN, OTA

stock standard solutions, respectively, and treated by the UAATPE-DLLME procedures described above. After being subjected to FTA derivatization, the mycotoxins were determined by HPLC coupled with DAD and FLD in series. Data obtained were fitted by linear regression according to the correlation between the peak area (A) and the concentration (C, ng/mL), and the results are shown in Table 1. Meanwhile, the limit of detection (LOD) and the limit of quantification (LOQ) were calculated according to signal-to-noise ratio of 3:1 (S/N = 3) and 10:1 (S/N = 10), respectively.

From Table 1, the results demonstrated that there was good linearity in the ranges of 0.5 – 100.0 ng/mL with correlation coefficients (R^2) higher than 0.9991. LODs and LOQs were in the range of 0.01563 – 0.5161 ng/mL and 0.05210 – 1.720 ng/mL, respectively. DLLME enrichment factors (EFs) were ranged from 12.2 to 21.1, exhibiting stronger enrichment ability to nine mycotoxins. The EF value of each mycotoxin was calculated by the following equation: $EF = C_b/C_0$ (C_0 and C_b were its initial concentrations and preconcentration concentration, respectively). By means of FLD detection, five aflatoxins, ZEN, and OTA could reach higher sensitivity instead of DAD.

Accuracy, precision, and stability

UAATPE-DLLME as sample pretreatment was coupled to HPLC–DAD-FLD for investigation of accuracy, precision, and stability. Accordingly, the recovery experiments were accomplished by spiking samples at 20.0 ng/g of nine mycotoxins standards to assess the accuracy of the proposed method. According to experimental results of six repetitions, precision and stability were evaluated by the intra-day and inter-day precisions with relative standard deviations (RSD). As shown in Table 2, the recoveries of nine mycotoxins ranged from 82.77 to 103.2%, and the RSDs of intra-day and inter-day precisions for these mycotoxins were 1.1 to 5.6% and 1.5 – 4.9%, respectively. The results revealed that the method achieved high accuracy, credible repeatability, and satisfactory stability, and suggested that the method could withstand the influence of the sample matrices.

Real sample analysis

According to the process in Fig. 1, the developed method was applied to the analysis of black beans, black sesame, lotus seed, apricot kernel, and litchi pulp to check the presence of the mycotoxins in medicinal and edible foods. Before HPLC analysis, the samples crushed were subjected to UAAPTE/SOD-DLLME procedures and FTA derivatization successively (See Fig. S3). Furthermore, the recovery experiments of five spiked samples were accomplished so as to further validate the accuracy. The results obtained from the analysis of five samples were summarized in Table 3.

As shown in Table 3 five different mycotoxins in the range of 2.364 – 5.714 μg/kg were observed from the above samples. The recoveries and RSDs of nine mycotoxins were respectively 82.31 – 104.3% and 1.8% – 4.2%, suggesting that the method was more accurate and reliable. Among them, AFB1 was detected respectively from lotus seed and apricot kernel, AFB1 and DON were found in black bean, and litchi pulp was contaminated by AFB1 and AFB2. Even more, AFG1, AFB2, DON, and ZEN were simultaneously observed in black sesame. The above results also demonstrated that these samples were easily exposed to the contamination of the mycotoxins due to the relative higher temperature and humidity environment in South China. However, monitoring of the mycotoxins is not regulated equally all over the world. Thus, it is imperative to strengthen control of mycotoxin contamination in order to ensure safety and quality of these foods.

Table 1 Regression equations, linear ranges, LODs and LOQs for the mycotoxins analyzed

Mycotoxin	Detector	λ (nm)	Regression equation	R^2	Linear range (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)	Enrichment factor
PAT	DAD	277	A = 103.34C + 352.98	0.9994	1.0–100.0	0.1341	0.4471	18.0
DON	DAD	218	A = 82.39C + 543.9	0.9991	1.0–100.0	0.2288	0.7628	13.9
ZEN	DAD	276	A = 93.55C + 72.585	0.9994	1.0–100.0	0.1622	0.5408	13.7
	FLD	Ex: 275, Em: 440	A = 9967C + 68.556	0.9993	0.5–50.0	0.04451	0.1484	16.9
OTA	DAD	334	A = 53.347C-53.689	0.9993	1.0–100.0	0.4195	1.398	13.6
	FLD	Ex: 333, Em: 460	A = 17523C-46991	0.9995	0.5–50.0	0.03457	0.1152	18.2
AFM_1	DAD	360	A = 44.038C-42.102	0.9993	1.0–100.0	0.5161	1.720	12.2
	FLD	Ex: 360, Em: 430	A = 27002C-51325	0.9991	0.5–50.0	0.02431	0.08103	14.8
AFB_1	FLD	Ex: 360, Em: 430	A = 30124C-3738.3	0.9993	0.5–50.0	0.02372	0.07907	14.9
AFG_1	FLD	Ex:360, Em: 430	A = 10017C-30606	0.9997	0.5–50.0	0.02961	0.09870	14.6
AFG_2	FLD	Ex: 360, Em: 430	A = 23522C-24491	0.9991	0.5–50.0	0.02671	0.08903	14.6
AFB_2	FLD	Ex: 360, Em: 430	A = 37355C-29180	0.9995	0.5–50.0	0.01563	0.05210	21.1

Table 2 Precision, stability and repeatability for detection of the mycotoxins analyzed

Mycotoxin	Spiked level (ng/g)	Intra-day precision(n = 6)										Inter-day precision(n = 6)									
		Black bean		Black sesame		Lotus seed		Apricot kernel		Litchi pulp		Black bean		Black sesame		Lotus seed		Apricot kernel		Litchi pulp	
		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
AFG_1	20.0	96.50	3.7	96.52	2.1	93.39	3.3	88.13	2.4	88.04	3.3	94.31	3.3	95.33	4.3	92.44	3.6	92.29	3.5	86.14	3.4
AFB_1	20.0	102.1	2.1	103.2	1.5	88.87	2.6	94.04	3.6	92.25	3.6	98.51	2.4	100.1	2.0	85.34	2.7	86.87	3.1	93.35	4.6
AFG_2	20.0	95.60	2.3	96.13	1.1	87.90	2.6	97.98	2.8	99.76	2.1	93.32	1.9	95.22	1.7	88.94	3.6	86.68	2.7	95.76	3.2
AFB_2	20.0	98.86	4.2	93.54	1.8	88.86	3.4	93.37	5.1	95.87	4.3	89.71	2.2	87.90	1.9	85.89	3.9	87.79	3.2	92.65	4.9
AFM_1	20.0	86.71	4.3	89.12	1.7	87.20	5.6	86.86	3.1	89.71	3.4	89.66	1.7	87.56	1.5	86.34	4.1	85.20	4.7	85.88	3.3
PAT	20.0	95.22	4.1	87.99	2.1	90.02	3.5	87.98	4.0	89.27	3.7	86.43	3.9	85.43	3.2	91.39	3.3	89.11	4.1	86.98	4.2
DON	20.0	90.29	2.9	85.23	3.2	92.17	3.5	90.91	2.7	96.43	2.9	84.75	3.5	82.77	3.8	91.07	3.2	90.47	3.6	89.81	2.8
ZEN	20.0	87.79	2.4	89.08	3.4	94.79	2.9	95.12	2.6	92.04	3.3	89.64	2.1	86.21	2.2	92.47	3.1	93.69	3.0	93.54	3.7
OTA	20.0	91.71	4.3	97.26	2.0	95.02	3.5	97.98	4.0	99.99	3.2	88.90	2.3	85.98	2.0	93.12	3.8	95.02	3.5	97.98	4.0

Table 3 The results of determination of mycotoxins in medicinal and edible foods (n = 3)

Mycotoxin	Black bean			Black sesame			Lotus seed			Apricot kernel			Litchi pulp		
	Content (μg/kg)	Recovery (%)	RSD (%)	Content (μg/kg)	Recovery (%)	RSD (%)	Content (μg/kg)	Recovery (%)	RSD (%)	Content (μg/kg)	Recovery (%)	RSD (%)	Content (μg/kg)	Recovery (%)	RSD (%)
AFG_1	–	94.05	2.3	3.531 ± 0.051	90.74	2.8	–	95.92	2.3	–	90.03	2.2	–	96.62	2.8
AFB_1	3.410 ± 0.077	92.63	1.9	–	96.02	2.0	2.364 ± 0.036	98.75	2.1	5.714 ± 0.028	103.5	1.8	4.731 ± 0.059	92.30	2.8
AFG_2	–	95.69	1.8	–	90.16	1.9	–	104.3	2.3	–	96.33	2.5		90.92	3.4
AFB_2	–	101.4	2.8	2.678 ± 0.039	93.84	4.2	–	94.55	2.8	–	94.49	1.9	2.664 ± 0.047	90.27	3.0
AFM_1	–	82.31	2.1		89.20	3.4	–	87.29	1.8	–	87.12	3.0	–	91.03	3.2
PAT	–	86.95	3.0	–	87.78	2.9	–	92.61	3.0	–	88.90	2.8	–	85.59	2.9
DON	3.551 ± 0.034	97.55	2.6	3.566 ± 0.069	98.99	3.0	–	97.09	2.9	–	96.12	2.3	–	100.1	2.3
ZEN	–	92.27	2.8	3.084 ± 0.014	90.09	2.8	–	94.79	2.5	–	93.74	2.5	–	95.37	3.4
OTA	–	92.09	2.4	–	96.70	2.5	–	96.77	2.7	–	97.82	2.4	–	97.46	3.3

Conclusions

In this work, ultrasonic-assisted UAATPE combining with SOD-DLLME was developed for extraction, purification, and enrichment of nine mycotoxins in medicinal and edible foods, followed by simultaneous determination using HPLC coupled to DAD and FLD in series. Compared to LLE method with mono-phase solvent, UAATPE/SOD-DLLME can not only extract target analytes from the samples but also remove impurities in the extract through twice successive biphasic extractions. Acetonitrile as the ATPS phase-forming component and the dispersive solvent could directly connect UAATPE with SOD-DLLME, and 1-dodecanol as extractant was easily solidified at 4 °C or in ice water. As a result, the sample preparation and preconcentration procedures were greatly simplified, the solidified extract was conveniently collected before and after FTA derivatization, and the sensitivity and selectivity of the mycotoxins were significantly improved. Using HPLC coupled to in-series connection of DAD and FLD improved high-throughput detection of the mycotoxins (Chinese Pharmacopoeia Commission 2020b; General Administration of Quality Supervision 2016; Shi et al. 2018), achieving simultaneous determination of nine mycotoxins in black bean, black sesame, lotus seed and apricot kernel and litchi. The proposed method was proved to be simple, feasible and reliable. AFB1, AFB2, AFG1, DON, and ZEN were respectively detected from the above samples, revealing that they were easily exposed to the contamination of the mycotoxins in South China. The results provided the evidence for legislation of monitoring the mycotoxins in medicinal and edible foods.

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Declarations

Ethics approval and consent to participate This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication Not applicable.

Conflict of interest All authors declare that they have no conflict of interest.

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