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Penicillic acid in fruits: Method development, validation by liquid chromatography-tandem mass spectrometry and survey in southern China

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Abstract

BACKGROUND: Penicillic acid (PA) is produced by *Aspergillus* spp. and *Penicillum* spp., which are common postharvest and storage fungi of fruits. PA can be of concern for human health due to its toxicity and high fruit consumption by the population. However, no data on PA occurrence in various fruits was yet reported. A quick, easy, cheap, effective, rugged, and safe (QuEChERS) approach for PA determination in various fruits was developed and applied to explore PA incidence in fruits.

RESULTS: The modified QuEChERS procedure with extraction by ethyl acetate and purification by multi-walled carbon nanotubes (MWCNTs), primary secondary amino (PSA), and octadecyl silane (C18) was established to determine PA in various fruits by high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). The average recoveries were 72.9-102.2% and relative standard deviations (RSDs) were 1.3-7.9%. A total of 161 fruits samples, including kiwi, apple, peach, grape, and mandarins/orange, were collected in southern China. The incidence of PA in fruits was 14.9%, and the levels of PA contamination were 0.200-0.596 $\mu g \cdot kg^{-1}$. Our results suggested that orange/mandarins, grape, and kiwi were favorable matrices for *Aspergillus* spp. and *Penicillum* spp. to produce PA, rather than peach and apple.

CONCLUSION: To the best of our knowledge, this is the first report about PA contamination in various fruits in China. Our study emphasized the necessity of the current established method, which could be used for continuous monitoring of PA and reducing the health risk to Chinese consumers.

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Keywords: mycotoxin, penicillic acid, fruits, occurrence, HPLC-MS/MS

Introduction

Mycotoxins are secondary metabolites mainly produced by specific filamentous fungi under appropriate conditions of temperature and humidity [1]. PA (chemical structure shown in Figure 1) was firstly discovered and named in 1913 [2]. *Penicillium arcuate* could yield the highest amount of PA even at low temperature [3]. PA ingestion could cause lots of toxicity symptoms, including hepatotoxic [4], cytotoxic to alveolar macrophages [5], carcinogenic in mice and rats [6], digitalis-like action on cardiac muscle [7], and dilating action on coronary and pulmonary vessels [7]. The potential human health hazard of PA was suggested when it was isolated from agricultural products.

PA was already detected in many agricultural products, including animal feed, corns, wheat, nuts, spices and fruits [3, 8-13]. Liang et al. reported PA contaminated chestnut samples from Shandong province of China and the concentration was 13.3-66.5 $\mu g \cdot kg^{-1}$ in positive samples [9]. PA was detected in dried paprika samples [3]. PA contamination has been approved in cracked, unpacked, and retail green table olives [10]. PA concentration was 7.3-17.9 $mg \cdot kg^{-1}$ in healthy parts of citrus fruits infected by *Penicillium* [11]. Producers of PA are especially common in *Penicillium* section *Viridicata* and in *Aspergillus* section *Circumdati* [12]. *Aspergillus* spp. and *Penicillum* spp. are the most common postharvest and storage fungi of fruits [13]. PA can be of concern for human health due to its toxicity and the high consumption of fruits by the population, so study on PA occurrence in various fruits available in the market will contribute to more realistic risk estimation.

Effective and reliable methods for mycotoxin detection are needed to facilitate the control and regulation of mycotoxin. Combined with sensitive and selective HPLC-MS/MS technology, QuEChERs extraction procedure was applied to detect PA in green coffee bean, chestnut, rice, maize, peanut, and raw coffee [9, 14-16]. QuEChERs approach involves organic solvents extraction step and a reversed-dispersive solid-phase extraction (r-DSPE) cleanup step. The r-DSPE step is applied to adsorb interfering substances in the matrices, including polar organic acids, sterols, pigment, and

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non-polar interfering substances [17]. An efficient r-DSPE cleanup of food extract is necessary to improve column lifetime and reduce the instrument downtime due to maintenance [18]. Unfortunately, the reported QuEChERs methods for PA pretreatment seldom had cleanup steps. Fruits are complex matrices (high sugar, fiber or pigment) which strongly influence accurate quantitation of PA and instrument/column maintenance. It is necessary to develop an optimal QuEChERs preparation method with efficient r-DSPE cleanup step for PA analysis in fruits.

The goal of this work was to develop and validate a rapid, sensitive, and accurate method for PA determination in fruits using HPLC-MS/MS (triple quadrupole). Apple, peach, grape, mandarins/orange, and kiwi were chosen as representative of pome fruits, stone fruits, berries and small fruits, citrus fruits, and miscellaneous fruitswith, respectively. The extraction and purification processes were optimized. The validated analytical method was applied to apple, peach, grape, mandarins/orange, and kiwi real samples in order to investigate PA occurrence in various fruits from southern China.

Materials and Methods

Chemicals and Apparatus

PA standard (purity≥99%) was purchased from J&K Scientific Co., Ltd. (Beijing, China). Standard stock solutions of PA (100 $mg \cdot L^{-1}$) were prepared in acetonitrile and stored at -20 °C. The working standard solutions were prepared daily. HPLC-grade acetonitrile was purchased from Merck (Darmstadt, Germany). HPLC-grade ammonium acetate, analytical grade anhydrous sodium chloride (NaCl), analytical grade ethyl acetate, and nanosized aluminum oxide powder (Al_2O_3, purity≥99.9%, 10 nm in diameter) were purchased from Aladdin Chemistry Co., Ltd. (Shanghai, China). Milli-Q deionized water (Millipore, Bedford, MA) was used throughout the analysis. PSA (40-60 μm in size), C18 (40-60 μm in size), and graphitized carbon black (GCB, 40-60 μm in size) were purchased from Bonna-Agela Technologies Inc. (Tianjin, China). MWCNTs (10-20 nm in outer diameter, 10-30 μm in length) were purchased from Nanjing XFNANO Materials Tech Co.,

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Ltd. (Jiangsu, China). Florisil (60-100 mesh) was supplied by Sinopharm Chemical Reagent Co. Ltd., China. Syringe filter (nylon, 0.22 μm in pore size, 13 mm in diameter) was purchased from Jinteng Experiment Equipment Co. Ltd. (Tianjin, China).

Centrifugation during sample preparation was performed in two different instruments: an Anke TDL-40B centrifuge equipped with a bucket rotor (8×50 mL, Shanghai, China) and a SIGMA 3K15 microcentrifuge equipped with angular rotor (24×2.0 mL, BMH Instruments Co. Ltd., China). A MX-F vortex mixer (DLAB Scientific Co., Ltd., Beijing, China) and a CSA-SA thermostatic gas bath oscillator (Saidilisi Experimental Analytical Instrument Factory, Tianjin, China) were used for preparing the samples. An EYELA rotary evaporator (Tokyo Rikakikai Co., Ltd., Japan) was used for solvent evaporation.

Collection of fruits samples

A total of 161 commercial fresh fruits were randomly purchased between July 2020 and September 2020. 73% of fruits were purchased from different local shops and supermarkets from Zhaoqing, Guangdong province, China. 27% of samples were bought from the online shops. Different fruit varieties were covered to ensure the survey was a representative study. The samples included 36 grape fruits, 34 kiwi fruits, 34 citrus fruits (mandarins and oranges), 27 peach fruits, and 30 apple fruits. Detail information on real samples, including place of purchase, fruit variety, number of each variety, and place of origin, was shown in Table S1. All collected fruits were visually in acceptable consumption conditions. Sample collection was carried out according to EU guidelines [19]. The samples size of fruits was at least 1 kg at each shop. The samples were kept in original package and sent back to lab within 4 h. Immediate homogenization was performed and the homogenized samples were pretreated using the optimized sample preparation procedures.

Sample Preparation

10 g (±0.02 g) of homogenized fruit sample (apple, peach, grape, kiwi, and orange flesh) was weighed into a 50 mL PTFE centrifuge tube. Thereafter, 10 mL of ethyl acetate was added and

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mechanical shaking was performed at 25°C for 30 min. After centrifugation at relative centrifugal force (RCF) 3802×g for 5 min, 7.5 mL of the clarified supernatant was evaporated until dryness at room temperature. 1.5 mL of acetonitrile was used to reconstitute the residue. 1.0 mL of the re-dissolved extract was introduced into a 2 mL micro-centrifuge tube containing 200 mg of C18, 10 mg of PSA, and 50 mg of MWCNTs. Immediate vortex shaking was performed for 30 s and the mixture was centrifuged at RCF 9998×g for 3 min. The upper layer was filtered through an autosampler vial with 0.22 μm syringe filters and analyzed directly by HPLC-MS/MS.

HPLC-MS/MS Analysis

The HPLC-MS/MS analysis was achieved using a Waters ACQUITY High-Performance LC system (Waters, Milford, MA, USA). Chromatographic separation was achieved on a Luna® Omega 1.6 μm PS C18 column (50 mm × 2.1 mm, Phenomenex, USA). The mobile phase at the flow rate of 0.2 $mL \cdot min^{-1}$ was the mixture of acetonitrile and 5 $mmol \cdot L^{-1}$ ammonium acetate (80/20, V/V). The injection volume was 3 μL and the column temperature was 30°C.

For MS/MS detection, a Waters T-QS mass spectrometer system (Waters, Milford, MA, USA) was used in negative electrospray ionization mode (ESI−) with the following parameters: interface voltages of capillary of 2.5 kV, desolvation temperature of 600 °C, and source temperature of 150 °C. The gas flow rates were 7.0 bar for nebulizing gas and 600 $L \cdot h^{-1}$ for desolvation gas, respectively. Multiple reaction monitoring (MRM) mode was used for the quantification and confirmation of PA with the parameters shown in Table S2.

Results and Discussion

Optimization of HPLC conditions

The composition of mobile phase is important for adjusting retention time, selectivity, and peak shape in HPLC separation [17]. 5 $mmol \cdot L^{-1}$ ammonium acetate was selected as water phase and organic phase was methanol or acetonitrile. Different acetonitrile percentage (90%, 80%, 70%, 60%) and different methanol percentage (90%, 80%, 70%) were optimized and the results were shown in

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Figure 2 and Figure S1, respectively. The mixture of 5 mmol·L^{-1} ammonium acetate and acetonitrile provided better shaped peak and higher response (Figure 2), rather than mixture of 5 mmol·L^{-1} ammonium acetate and methanol (Figure S1). When the mobile phase was the mixture of acetonitrile-5 mmol·L^{-1} ammonium acetate (8/2, V/V), satisfactory peak appearance and high peak response were achieved for PA. Different column temperature (20℃, 25℃, 30℃, 35℃, 40℃, 45℃) was set to investigate its influence on the separation. The peak shape was almost the same at different column temperature. Considering peak response (Figure S2), column temperature of 30℃ was selected.

Optimization of MS/MS conditions

PA is active acid which contains -COOH functional group and tends to lose H^+ in ionization. However, PA also exists in two tautomeric forms with small energy differences (Figure 1) [20, 21]. The two forms of penicillic acid can be readily interconverted by suitable physical treatment, like grinding in "Nujol" [21]. Therefore, PA exhibited good ionization at both polarities. Casquete [3] and Vaclavik [16] detected PA in positive ESI mode (m/z 171 as precursor ion). Liang [9] and Kokkonen [22] detected PA in negative mode (m/z 169 as precursor ion). PA ionization mode was optimized in our study. When PA ionized with negative mode (ESI-), higher response was obtained than in positive mode (Figure S3). So ESI- was chosen to monitor the precursor ions. The precursor ion (m/z 169.03, $[M-H]^-$) with the highest relative intensity in full scan was selected, and the product ion (m/z 109.836) was chosen as the quantitative ion.

Optimization of sample pretreatment

Efficient sample pretreatment can promote the reliable isolation of target mycotoxin and serve to decrease matrix effects [9]. Our study aimed to establish a modified QuEChERs approach with efficient r-DSPE cleanup step for PA analysis in fruits. In order to improve recoveries and reduce matrix effects, several experimental parameters affecting analytes partition in fruit matrices were investigated, including extraction solvent, the combination of r-DSPE cleanup sorbent and the amount of cleanup sorbent.

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Extraction solvent optimization

Two extraction solvents (acetonitrile, ethyl acetate) were tested and the experiment was performed by adding 10 mL of extraction solvent to 10 g of blank kiwi samples at PA spiked level of 0.02 $mg \cdot L^{-1}$. The recoveries of PA extracted by acetonitrile (ACN) and ethyl acetate (EtOAc) were (94.4±7.4) % and (86.7±1.3) %, respectively. PA stock solution (10 $mg \cdot L^{-1}$) was diluted with ACN extracts and EtOAc extracts of blank kiwi sample to obtain 0.1 $mg \cdot L^{-1}$ of ACN-matrix-solution and 0.1 $mg \cdot L^{-1}$ of EtOAc-matrix-solution, respectively. The matrix solutions were analyzed by HPLC-MS/MS. As shown in Figure S4, the peak area of EtOAc-matrix-solution was 2347±14, which was approximately twice that of ACN-matrix-solution (1226±95). EtOAc as extraction solvent could contribute to higher sensitivity of PA. As a consequence, EtOAc was chosen as extraction solvent in this study.

r-DSPE conditions optimization

For the cleanup, the following r-DSPE sorbents and mixtures of them were evaluated: PSA, GCB, C18, MWCNTs, nanosized Al_2O_3, and Florisil. PSA is a weak anion exchange sorbent that retains carboxylic acids via hydrogen bond interaction [23]. C18 is a nonpolar sorbent that more effectively retains non-polar compounds from the extract [24]. GCB is used for pigment and sterols removal [24]. MWCNTs has superior performance on pigment and other interfering substances cleanup in the complex food matrices [23, 25, 26]. Al_2O_3 has been used for lipid cleanup in r-DSPE step [27] and Florisil is a commonly used solid phase extraction adsorbent.

Firstly, mixture of 20 mg GCB and 50 mg C18 and mixture of 20 mg MWCNTs and 50 mg C18 were used as r-DSPE sorbents to compare the cleanup performance of GCB and MWCNTs. The average recoveries and peak areas of matrix solutions were almost the same for purification by GCB+C18 mixture and MWCNTs+C18 mixture (Table S3). As shown in Figure 3, mixture of MWCNTs and C18 displayed better pigment cleanup performance than GCB and C18 mixture. Therefore, MWCNTs was chosen as a suitable alternative to GCB in r-DSPE cleanup step.

Orthogonal array experimental design (OAED) is a highly efficient, economical and easy-to-

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use multi-factor experimental design method to research a target with multiple factors and levels [28]. The OAED is used to select some representative points from the full factorial experiment for conducting experiments on the basis of orthogonality [28]. Thus, the results equivalent to a full factorial experiment design can be obtained with the least number of experiments [28]. In this study, OAED was applied for the first time to screen the r-DSPE step to optimize sorbent combination and sorbent amount. PSA, C18, MWCNTs, Al_2O_3, and Florisil were influence factors. Sorbent amount was factor level. PA average recovery and PA peak area of matrix solution were considered as experimental indices. Single-factor experiment was conducted to evaluate sorbent amount used in OAED. The experiment design and results were shown in Supplemental Materials. Preferably on the basis of single-factor experiments, the influence factors and the corresponding level values selected were summarized in Table 1. The factors of MWCNTs, PSA, Al_2O_3, Florisil, and C18 were marked as A, B, C, D, and E. The four factor levels were indicated by levels 1-4. Therefore, the minimum orthogonal array L_{16} (4^5) was selected from the orthogonal table to arrange the orthogonal design subsequently. Assignments of factors and levels of screening between number of values using a L_{16} (4^5) matrix were presented in Table 2. The run order of the trials was arbitrary to avoid any personal or subjective bias. The corresponding consequences measured were given in Table 2. The orthogonal comprehensive mark analysis was used to optimize sorbent combination and sorbent amount. Two indices were converted into their membership degrees. Membership degree (MD) was calculated as:

$$membership\ degree\ of\ index = \frac{index_{x,j} - index_{min,j}}{\mathrm{index}_{max,j} - index_{min,j}}$$

where *x* represented the trial number, from 1 to 16; *j* was the index of PA average recovery or PA peak area of matrix solution; $index_{min,j}$ indicated the minimum value of PA average recovery or PA peak area of matrix solution; $index_{max,j}$ indicated the maximum value of PA average recovery or PA peak area of matrix solution. The calculated MD results were listed in Table 2. To a certain degree, the peak area of matrix solution represented the sensitivity of the analytical method, which was equally important to PA average recovery in our analytical result. The index weight was 0.5 for

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recovery and 0.5 for peak area. Therefore, the comprehensive mark for each trial was calculated as:

$$comprehensive\ mark = MD\ of\ recovery \times 0.5 + MD\ of\ peak\ area \times 0.5$$

Range analysis was adopted to estimate the influence degree of each factor to the results. Range value (R) can be calculated as:

$$R_i = \max(K_i) - \min(K_i)$$

where *i* represented the factor levels, from 1 to 4; R_i denoted the range value of factor *i*; K_i indicated the total value of the corresponding comprehensive mark at all level *i*. The larger the range value of a factor, the greater the effect of the factors on the overall result, and this factor was more sensitive to the experimental results. As evidence from Table 2, the range value was $R_{\mathrm{MWCNTs}} > R_{\mathrm{PSA}} > R_{\mathrm{Al2O3}} > R_{\mathrm{Florisil}} > R_{\mathrm{C18}}$. That's why the order of influence degree (from the most to least) was MWCNTs > PSA > Al_2O_3 > Florisil > C18 for the experimental result. The effect of MWCNTs on the experimental result was the largest, followed by PSA, then by Al_2O_3, Florisil, and C18.

The optimal scheme referred to the combination of the optimal levels of various factors within the experimental scope. The determination of the optimal level of each factor was related to the test index. If the larger index is better, the level that makes the index larger should be selected. In this study, the larger PA average recoveries and the larger average peak area of matrix solution were the better. Therefore, the optimum scheme was mixed 40 mg of MWCNTs, 10 mg of PSA, 5 mg of Al_2O_3, 5 mg of Florisil, and 50 mg of C18, which was the optimum r-DSPE condition based on orthogonal analysis. However, the optimum experimental condition was not included in the 16 trials of orthogonal experiments. In order to verify whether the optimum scheme can provide good accuracy and high sensitivity, recovery studies on kiwi, orange flesh, apple, grape, and peach with spiked level of 20 μg·kg^{-1} were conducted according to the optimum scheme and trial No. 9, 12, and 14. Method 1-4 shown in Figure 4 referred to the optimum scheme, trial No. 9, trial No. 12, and trial No. 14, respectively. The sorbent combination and sorbent amount of method 1-4 were listed in the legend of Figure 4. To select the most efficient method, the r-DSPE cleanup method that showed a recovery within the range of 70-110% and relatively larger peak area were considered.

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Thus, as demonstrated in Figure 4, good results were achieved using method 2 followed by r-DSPE cleanup by mixture of 50 mg MWCNTs, 10 mg PSA, and 200 mg C18. Even though the optimum scheme obtained from orthogonal analysis provided the highest sensitivity (the largest peak area of matrix solution), average recoveries for apple, orange, kiwi, and peach were lower than 70%. Moreover, method 2 was much easier to handle than the other three methods. Therefore, the r-DSPE step, using 50 mg of MWCNTs, 10 mg of PSA, and 200 mg of C18 as cleanup sorbents, was selected to be validated.

Method validation

Validation was performed according to EU Commission Regulation (EC) No. 401/2006 [19] and the parameters were also taken into account to ensure the reliability of the results.

Linearity

Stock solutions of PA (10 mg·L^{-1}) were diluted with acetonitrile (solvent), the final extract solution of PA-free kiwi, apple, grape, peach, and orange in sequence to obtain the calibration levels of concentrations of 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50, and 100 μg·L^{-1}. The calibration curves were fitted to a linear function and the results are summarized in Table 3. Linearity was assessed by calculating the determination coefficient (*r*) of the calibration curves obtained from standards in blank fruit extract and in organic solvent. Good linearity with *r*-values > 0.999 was realized over the experimental concentration ranges.

Matrix effects

Matrix effects caused by the co-extraction of matrix components are common and inevitable in HPLC-MS/MS [9]. The presence of matrix components can enhance or suppress the response of the mycotoxins during the ionization process and may, therefore, interfere with quantification and lead to incorrect results [9]. The prediction of matrix effects is influenced by several factors, such as the target compound, matrix type, and matrix/mycotoxin concentration ratio [29]. Sample treatment, chromatographic conditions, mass spectrometric instrumentation, and ionization conditions also influence the extent and nature of matrix effects [29, 30].

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Matrix effects were calculated based on the slopes of the calibration curves obtained from standard solutions prepared in blank fruit extract and in organic solvent according to the equation below.

$$matrix\ effect\ (\%) = \left[\left(\frac{a}{b}\right) - 1\right] \times 100$$

where *a* represented slope of the calibration curve obtained from standard solutions in blank fruit extract; *b* represented slope of the calibration curve obtained from standard solutions in organic solvent. In general, matrix effects can be classified into soft (suppression or enhancement of 0-20%), medium (suppression or enhancement of 20-50%), and high (suppression or enhancement >50%) matrix effects [30]. As summarized in Table 4, medium matrix effects (detector response suppression) occurred with PA in the five tested fruit matrices. Thus, it was advisable to use matrix-matched calibration standards to avoid inaccurate quantification of analytes for which the matrix effects exceed the limit of 20%.

Accuracy and precision

To evaluate accuracy and precision of the method, a recovery study was performed by applying the optimized methods to residue free kiwi, apple, grape, peach, and orange samples which were spiked at three concentration levels of 0.2, 2, and 20 μg·kg^{-1} in five replicates. Results from fortification studies were statistically analyzed using Excel to calculate average recovery of PA and its relative standard deviations (RSD, %). Table 4 represented the recoveries and RSDs of PA in fruit samples. The average recoveries were 72.9-102.2% and RSDs were 1.3-7.9%. Overall, the results demonstrated good recovery values and satisfied the analysis requirements of Commission Regulation (EC) 401/2006 [19].

Limit of detection (LOD) and Limit of quantification (LOQ)

The sensitivity of the method was evaluated by LOD and LOQ. LOD was experimentally determined by spiked blank fruit extracts with PA. The LOD was defined as the lowest concentration of analyte that could be differentiated of the matrix signal with a signal-to-noise ratio (S/N) greater than 3 [9, 15, 16, 31]. The lowest concentration levels in matrix matched calibration curve were injected

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in six replicative to confirm the method's LOD with S/N > 3. The LODs were 0.1-0.2 μg·L^{-1} for our method (Table 4). The LOQ was based on the trueness and precision data, obtained via the recovery determination and was defined as the lowest validated spike level meeting the requirement of a recovery within the range of 70-120 % and RSD≤20% [24, 31]. It can be seen that LOQs were 0.2 μg·kg^{-1} for all fruit matrices (Table 4).

Comparison with the earlier developed methods

The proposed method was compared with the earlier developed methods for PA analysis in food (Table 5). From the published reference data, the recoveries and RSDs of PA in different food matrices ranged from 70% to 109% and 2% to 18%, respectively. Reasonable performance in recoveries and RSDs was achieved in MWCNTs-PSA-C18-based r-DSPE cleanup method, with recoveries of 72.9-102.2% and RSDs of 1.3-7.9%. Meanwhile, much lower LOQs of 0.2 μg·kg^{-1} and shorter instrumental analysis time were obtained when compared with other reported method. The proposed method showed good precision, high sensitivity, and time-saving advantage.

Occurrence of PA in commercial fruits samples in China

Fruits are susceptible to PA producing fungi under high moisture/humid environmental condition during postharvest and storage process, so investigation of PA contamination in various fruits is needed, especially in humid conditions in southern China. The HPLC-MS/MS method was used for screening of PA in 161 fruits samples. According to the results presented in Table 6, the present study revealed an incidence of positive samples of 14.9% (24/161) and the levels of PA contamination in the range of 0.200-0.596 μg·kg^{-1}. The PA level was lower than LOQ for all the peach and apple samples. Regarding the analyzed commodities, percentage of positive samples were 14.7%, 16.7%, and 38.2% in kiwi, grape, and citrus fruits, respectively. The results demonstrated that the highest levels of PA contamination were 0.420, 0.389, and 0.0.596 μg·kg^{-1} for kiwi, grape, and citrus fruits, respectively. The chromatograms of PA detected in positive grape samples were shown in Figure S6. Hallas-Møller et al. reported that the concentration of PA was determined to be 1095 mg·kg^{-1} in wheat samples [32]. Liang et al. reported that the chestnut samples from Shandong

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province of China were contaminated with PA and PA concentration was 13.3-66.5 μg·kg^{-1} in the positive chestnut samples [9]. Compared to the contaminations in cereals and nuts, the obviously lower incidences of PA in the original fruit were observed. It was noteworthy that the grapes, oranges/mandarins, and kiwi were frequently contaminated with PA, and there was a need to improve prevention and control strategies during pre- and post-harvest procedures.

Conclusions

This study developed a reliable and sensitive method using MWCNTs, PSA, and C18 as r-DSPE cleanup sorbents to determine PA concentration in various fruits, which was combined with HPLC-MS/MS detection. Sample pre-treatment was optimized. The average recoveries of PA were between 72.9% and 102.2% and consistent RSDs were 1.3-7.9%. The developed method was applied for a survey of PA occurrence in kiwi, apple, peach, grape, and mandarins/orange in southern China. Overall, 14.9% of total samples were positive samples and the levels of PA contamination in the range of 0.200-0.596 μg·kg^{-1}. The survey results strongly suggested that the grape, kiwi, and orange/mandarins were favorable matrices for *Aspergillus* spp. and *Penicillum* spp. producing PA, rather than peach and apple. Our study emphasized the necessity of the current established method, which could be used for continuous monitoring of PA and reducing the health risk to consumers in China.

References

1 Ji XF, Li R, Yang H, Qi PP, Xiao YP, Qian MR. Occurrence of patulin in various fruit products and dietary exposure assessment for consumers in China. *Food Control* **78**:100-107 (2017).

2 Alsberg C, Black OF. Contributions to the study of maize deterioration: Biochemical and toxicological investigations of *Penicillium puberulum* and *Penicillium stoloniferum*. US: Government Printing Office. (1913).

3 Casquete R, Rodriguez A, Hernandez A, Martin A, Bartolome T, Cordoba JJ, Cordoba GM. Occurrence of toxigenic fungi and mycotoxins during smoked paprika production. *J Food Prot* **80**: 2068-2077 (2017).

4 Veselý D, Vesela D. Use of chick embryos for prediction of embryotoxic effects of mycotoxins in

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mammals. *Vet Med* **36**:175-181 (1991).

5 Pandiyan V, Nayeem M, Nanjappan K, Ramamurti R. Penicillic acid as Na^+, K^+ and Ca^{2+} channel blocker in isolated frog's heart at toxic levels. *Indian J Exp Biol* **28**:295-296 (1990).

6 Dickens E, Jones HEH. Further studies on the carcinogenic action of certain lactones and related substances in the rat and mouse. *Brit J Cancer* **19**:392-403 (1965).

7 Scott PM, Somers E. Stability of patulin and penicillic acid in fruit juices and flour. *J Agr Food Chem* **16**: 483-485 (1968).

8 Ling SM, Li XL, Zhao Q, Wang RZ, Tan T, Wang SH. Preparation of monoclonal antibody against penicillic acid (PA) and its application in the immunological detection. *Food Chem* **319**: 126505 (2020).

9 Liang JY, Dong YJ, Yuan XX, Fan LX, Zhao SC, Wang L. Fast determination of 14 mycotoxins in chestnut by dispersive solid-phase extraction coupled with ultra high performance liquid chromatography-tandem mass spectrometry. *J Sep Sci* **42**:2191-2201 (2019).

10 Khalil AMA, Hashem AH, Abdelaziz AM. Occurrence of toxigenic Penicillium polonicum in retail green table olives from the Saudi Arabia market. *Biocat Agr Biotech* **21**:101314 (2019).

11 Yu X, Feng T, Pang J, Zhang BC. Study on penicillin toxins in postharvest citrus. *Chinese Food Sci* **19**:11-13 (1998).

12 Frisvad JC. A critical review of producers of small lactone mycotoxins: patulin, penicillic acid and moniliformin. *World Mycotoxin J* **11**:73-100 (2018).

13 Pallarés N, Carballo D, Ferrer E, Fernández-Franzón M, Berrada H. Mycotoxin dietary exposure assessment through fruit juices consumption in children and adult population. *Toxins* **11**:684 (2019).

14 Manizan AL, Oplatowska-Stachowiak M, Piro-Metayer I, Campbell K, Koffi-Nevry R, Elliott C, Akaki D, Montet D, Brabet C. Multi-mycotoxin determination in rice, maize and peanut products most consumed in Cote d'Ivoire by UHPLC-MS/MS. *Food Control* **87**:22-30 (2018).

15 Reichert B, Kok dA, Pizzutti RI, Scholten J, Cardoso DC, Spanjer M. Simultaneous determination of 117 pesticides and 30 mycotoxins in raw coffee, without clean-up, by LC-ESI-MS/MS analysis. *Anal Chim Acta* **1004**:40-50 (2017).

16 Vaclavik L, Vaclavikova M, Begley HT, Krynitsky JA, Rader IJ. Determination of multiple mycotoxins in dietary supplements containing green coffee bean extracts using ultrahigh-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). *J Agric Food Chem* **61**:4822-4830 (2013).

17 Guan W, Xu PJ, Wang K, Song Y, Zhang HY. Determination and study on dissipation of 1-naphthylacetic acid in garlic and soil using high performance liquid chromatography-tandem

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mass spectrometry. *Food Chem Toxicol* **49**:2869-2874 (2011).

18 Zhao LM, Szakas T, Churley M, Lucas D. Multi-class multi-residue analysis of pesticides in edible oils by gas chromatography-tandem mass spectrometry using liquid-liquid extraction and enhanced matrix removal lipid cartridge cleanup. *J Chromatogr A* **1584**:1-2 (2019).

19 European Commission. (2006b). Commission Regulation (EC) No. 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. Official Journal of the European Communities of 9 March 2006, L 70, 12-34.

20 Birkinshaw JH, Oxford AE, Raistrick H. Studies in the biochemistry of micro-organisms: Penicillic acid, a metabolic product of *Penicillium puberulum* Bainier and *P. cylopium* Westling. *Biochem J* **30**: 394-411 (1936).

21 Munday C. Tautomerism of penicillic acid. *Nature* **163**: 443-444 (1949).

22 Kokkonen MK, Jestoi MN. A multi-compound LC-MS/MS method for the screening of mycotoxins in grains. *Food Anal Methods* **2**:128-140 (2009).

23 Zhao PY, Wang L, Zhou L, Zhang FZ, Kang S, Pan CP. Multi-walled carbon nanotubes as alternative reversed-dispersive solid phase extraction materials in pesticide multi-residue analysis with QuEChERS method. *J Chromatogr A* **1225**:17-25 (2012).

24 Guan W, Li CS, Liu X, Zhou SJ, Ma YQ. Graphene as dispersive solid phase extraction materials for pesticides LC-MS/MS multi-residue analysis in leek, onion and garlic. *Food Addit Contam A* **31**: 250-261 (2014).

25 Dong MF, Si WS, Jiang KQ, Nie DX, Wu YJ, Zhao ZH, Saeger DS, Han Z. Multi-walled carbon nanotubes as solid-phase extraction sorbents for simultaneous determination of type A trichothecenes in maize, wheat and rice by ultra-high performance liquid chromatography-tandem mass spectrometry. *J Chromatogr A* **1423**:177-182 (2015).

26 Xu X, Xu XY, Han M, Qiu ST, Hou X. Development of a modified QuEChERS method based on magnetic multiwalled carbon nanotubes for the simultaneous determination of veterinary drugs, pesticides and mycotoxins in eggs by UPLC-MS/MS. *Food Chem* **276**:419-426 (2018).

27 Anagnostopoulos C, Miliadis GE. Development and validation of an easy multiresidue method for the determination of multiclass pesticide residues using GC-MS/MS and LC-MS/MS in olive oil and olives. *Talanta* **112**:1-10 (2013).

28 Li J, Yang FB, Zhang HG, Wu Z, Tian YM, Hou XC, Xu YH, Ren J. Comparative analysis of different valve timing control methods for single-piston free piston expander-linear generator via an orthogonal experimental design. *Energy* **195**: 116966 (2020).

29 Desmarchelier A, Oberson JM, Tella P, Gremaud E, Seefelder W, Mottier P. Development and

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comparison of two multiresidue methods for the analysis of 17 mycotoxins in cereals by liquid chromatography electrospray ionization tandem mass spectrometry. *J Agric Food Chem* **58**: 7510-7519 (2010).

30 Zhang B, Chen X, Han SY, Li M, Ma TZ, Sheng WJ, Zhu X. Simultaneous analysis of 20 mycotoxins in grapes and wines from Hexi corridor region (China): Based on a QuEChERS-UHPLC-MS/MS method. *Molecules* **23**: 1926 (2018).

31 Renata PL, Fabiano ASO, Fernando DM, Mauro LGO, Gilsara S. Multiresidue analysis of pesticide in peanuts using modified QuEChERS sample preparation and liquid chromatography-mass spectrometry detection. *Anal Methods* **7**: 4734-4739 (2015).

32 Hallas-Møller M, Nielsen KF, Frisvad JC. Production of the fusarium mycotoxin moniliformin by penicillium melanoconidium. *J Agric Food Chem* **64**:4505-4510 (2016).

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Conflict of interest statement

All authors have declared no conflict of interest.

Figure Legends

Figure 1 Two tautomeric forms of penicillic acid: (a) 3-methoxy-5-methyl-4-oxo-2,5-hexadienoic acid; (b) 5-hydroxy-5-isopropenyl-4-methoxy-2(5H)-furanone.

Figure 2 Multiple reaction monitoring (MRM) chromatograms of penicillic acid (PA) at 100 $\mu g \cdot L^{-1}$ obtained by HPLC-MS/MS analysis at different proportions (60%, 70%, 80%, 90%) of acetonitrile as organic part of mobile phase.

Figure 3 Photography of pigment-cleanup performance by different cleanup sorbents: (a) extract for blank kiwi sample without reversed-dispersive solid-phase extraction (r-DSPE) cleanup step; (b) extract for blank kiwi sample with r-DSPE cleanup by 50 mg octadecyl silane (C18) and 30 mg graphitized carbon black (GCB); (c) extract for blank kiwi sample with r-DSPE cleanup by 50 mg C18 and 30 mg multi-walled carbon nanotubes (MWCNTs).

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Figure 4 Penicillic acid (PA) average recoveries and peak area of matrix matched solution prepared with blank fruit extract with r-DSPE cleanup by different sorbents. Method 1 referred to the optimum scheme with cleanup step by 40 mg of MWCNTs, 10 mg of PSA, 5 mg of Al_2O_3, 5 mg of Florisil, and 50 mg of C18. Method 2 referred to trial No. 9 in orthogonal experiment with cleanup step by 50 mg of MWCNTs, 10 mg of PSA, and 200 mg of C18. Method 3 referred to trial No. 12 in orthogonal experiment with cleanup step by 50 mg of MWCNTs, 5 mg of Al_2O_3, 5 mg of Florisil, and 100 mg of C18. Method 4 referred to trial No. 14 in orthogonal experiment with cleanup step by 30 mg of MWCNTs, 5 mg of PSA, 5 mg of Florisil, and 150 mg of C18.

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Table 1 Five influence factors (marked as Factor A, B, C, D, and E) and the corresponding four factor level values[a] selected for orthogonal array experimental design

Factor levels	Factor A	Factor B	Factor C	Factor D	Factor E
	MWCNTs (mg)	PSA (mg)	Al_2O_3 (mg)	Florisil (mg)	C18 (mg)
1	20	10	15	5	50
2	40	5	5	10	200
3	50	15	0	15	100
4	30	0	10	0	150

MWCNTs: multi-walled carbon nanotubes, PSA: primary secondary amino, Al_2O_3: nanosized aluminum oxide powder, C18: octadecyl silane.

[a] Factor level values mean the amount of each cleanup sorbent.

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Table 2 Assignments of factors and levels of screening between number of values using a L_{16} (4^5) matrix, the orthogonal experimental results including two indices under direct observation analysis, calculated membership degree, calculated comprehensive mark, range value (*R*), influence degree of factors, and optimum scheme.

Trial No.	Factors					Indices		Membership degree (MD)		Comprehensive mark
	MWCNTs (mg)	PSA (mg)	Al_2O_3 (mg)	Florisil (mg)	C18 (mg)	Average recoveries (%)	Peak area of matrix solution[a]	MD of average recoveries	MD of peak area	
1	20	10	15	5	50	55.7	4904	0.22	1.00	0.61
2	20	5	5	10	200	61.7	4105	0.45	0.63	0.54
3	20	15	0	15	100	55.7	4425	0.22	0.78	0.50
4	20	0	10	0	150	66.2	3244.5	0.62	0.23	0.42
5	40	10	5	15	150	56.9	4259.5	0.27	0.70	0.48
6	40	5	15	0	100	57.8	4102.5	0.30	0.63	0.46
7	40	15	10	5	200	55.1	4259.5	0.20	0.70	0.45
8	40	0	0	10	50	68.1	3033.5	0.69	0.13	0.41
9	50	10	0	0	200	70	3185.5	0.76	0.20	0.48
10	50	5	10	15	50	58.4	3926	0.32	0.55	0.43
11	50	15	15	10	150	52.7	4443	0.11	0.79	0.45
12	50	0	5	5	100	76.4	2751.5	1.00	0.00	0.50
13	30	10	10	10	100	58.4	3900.5	0.32	0.53	0.43
14	30	5	0	5	150	69.7	3042	0.75	0.13	0.44
15	30	15	5	0	50	60	3956	0.38	0.56	0.47
16	30	0	15	15	200	49.8	4450	0.00	0.79	0.39
K_1	2.07	2.00	1.92	2.00	1.93					
K_2	2.29	1.88	1.99	1.82	1.86					
K_3	1.38	1.87	1.83	1.81	1.89					
K_4	1.74	1.73	1.74	1.84	1.80					
R	0.91	0.28	0.26	0.19	0.13	$R_{MWCNTs} > R_{PSA} > R_{Al2O3} > R_{Florisil} > R_{C18}$				

Influence degree of factors: MWCNTs > PSA > Al_2O_3 > Florisil > C18

Optimum scheme: 40 mg of MWCNTs, 10 mg of PSA, 5 mg of Al_2O_3, 5 mg of Florisil, 50 mg of C18

MWCNTs: multi-walled carbon nanotubes, PSA: primary secondary amino, Al_2O_3: nanosized aluminum oxide powder, C18: octadecyl silane.

[a] matrix solution means penicillic acid standard solution prepared in blank kiwi extract with r-DSPE cleanup by different sorbents. The concentration of matrix solution was 100 $\mu g \cdot L^{-1}$.

K_1, K_2, K_3, and K_4 indicated the total value of the corresponding comprehensive mark at all level 1, level 2, level 3, and level 4, respectively.

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Table 3 Liner range (μg·L^{-1}), correlation equation, and correlation coefficients (*r*) within a certain range for the calibration curves prepared with standards in blank fruit extract or in organic solvent.

	Organic solvent	Kiwi	Grape	Peach	Orange	Apple
Linear range in μg·L^{-1}	0.1-100	0.2-100	0.2-100	0.1-100	0.2-100	0.2-100
Correlation equation	y = 63.14x-5.53	y=31.52x-11.11	y=43.81x-47.17	y=40.47x-40.31	y=40.15x-20.15	y=40.51x-34.32
Correlation coefficients (*r*)	0.9999	0.9996	0.9994	0.9991	0.9998	0.9997

Table 4 Average recoveries (%), RSDs (%) (n=5), matrix effects (%), limit of detection (LOD), and limit of quantification (LOQ) for penicillic acid determination by HPLC-MS/MS in various fruits samples

Fruit	Fortification level (μg·kg^{-1})						Matrix effects (%)	LOD (μg·L^{-1})	LOQ (μg·kg^{-1})
	0.2		2		20				
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)			
Kiwi	84.4	7.9	82.4	4.6	72.9	2.9	-49.6	0.2	0.2
Grape	91.7	4.8	102.2	4.3	83.3	2.7	-30.7	0.2	0.2
Peach	76.9	5.3	83.8	3.9	77.8	1.3	-35.9	0.1	0.2
Orange	77.4	7.8	87.2	6.3	79.2	2.1	-36.4	0.2	0.2
Apple	101.7	3.2	88.7	5.7	80.3	1.4	-35.8	0.2	0.2

Table 5 Comparison with the earlier developed methods

Matrices	Determination method	Total instrumental analysis time	Recovery (RSDs)	LOQ (μg·kg^{-1})	Matrix effect	Reference
Chestnut	HPLC-MS/MS	14 min	80-91% (7.6-10.2%)	2	28%	9
Rice, maize, peanut	HPLC-MS/MS	13.5 min	No data	10	No data	14
Raw coffee	LC-MS/MS	13 min	89-104% (11-18%)	100	-30%	15
Green coffee bean	HPLC-MS/MS	13 min	105-109% (8-11%)	100	-40% to -89%	16
Wheat, barley, and oat	HPLC-MS/MS	30 min	70-108% (2-15%)	35-70	-45% to -75%	22
Kiwi, grape, citrus fruits, peach, apple	HPLC-MS/MS	2 min	72.9-102.2% (1.3-7.9%)	0.2	-30.7% to -49.6%	This method

LOQ: limit of quantification

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Table 6 Occurrence of penicillic acid in various fruits collected in China

Fruits	Positive/total	Positive detection rate (%)	Minimum ($\mu g \cdot kg^{-1}$)	Maximum ($\mu g \cdot kg^{-1}$)	Mean of positive samples ($\mu g \cdot kg^{-1}$)	Mean of all samples ($\mu g \cdot kg^{-1}$)
Kiwi	5/34	14.7	0.204	0.420	0.304	0.0448
Grape	6/36	16.7	0.229	0.389	0.280	0.0411
Citrus fruits	13/34	38.2	0.200	0.596	0.384	0.136
Peach	0/27	0	<LOQ	<LOQ	<LOQ	<LOQ
Apple	0/30	0	<LOQ	<LOQ	<LOQ	<LOQ

Positive/total: the number of positive fruit samples/the number of total fruit samples.

Minimum: minimum concentration of penicillic acid among the positive fruit samples.

Maximum: maximum concentration of penicillic acid among the positive fruit samples.

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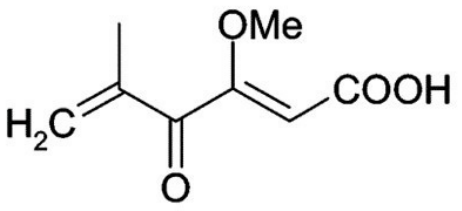

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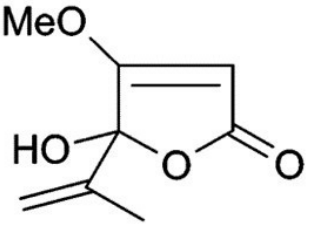

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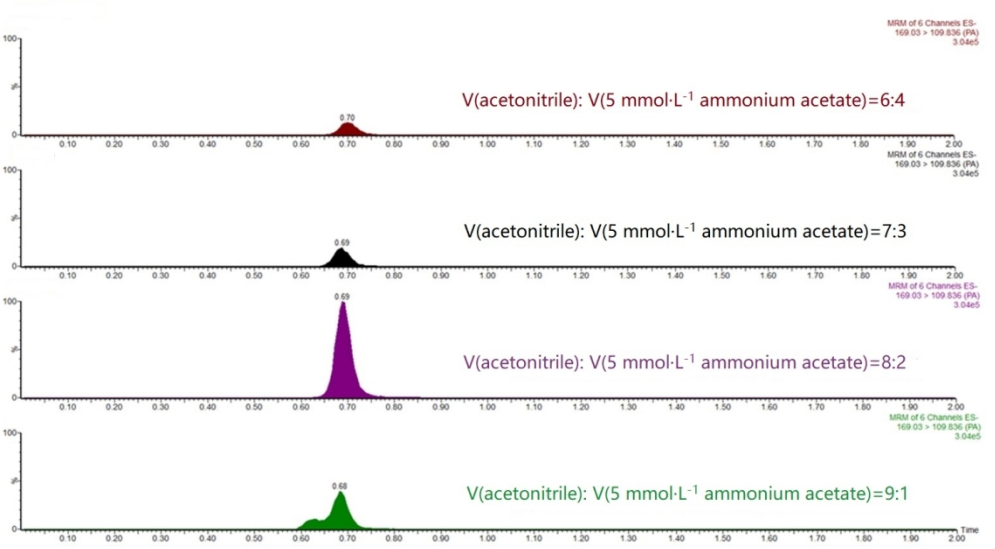

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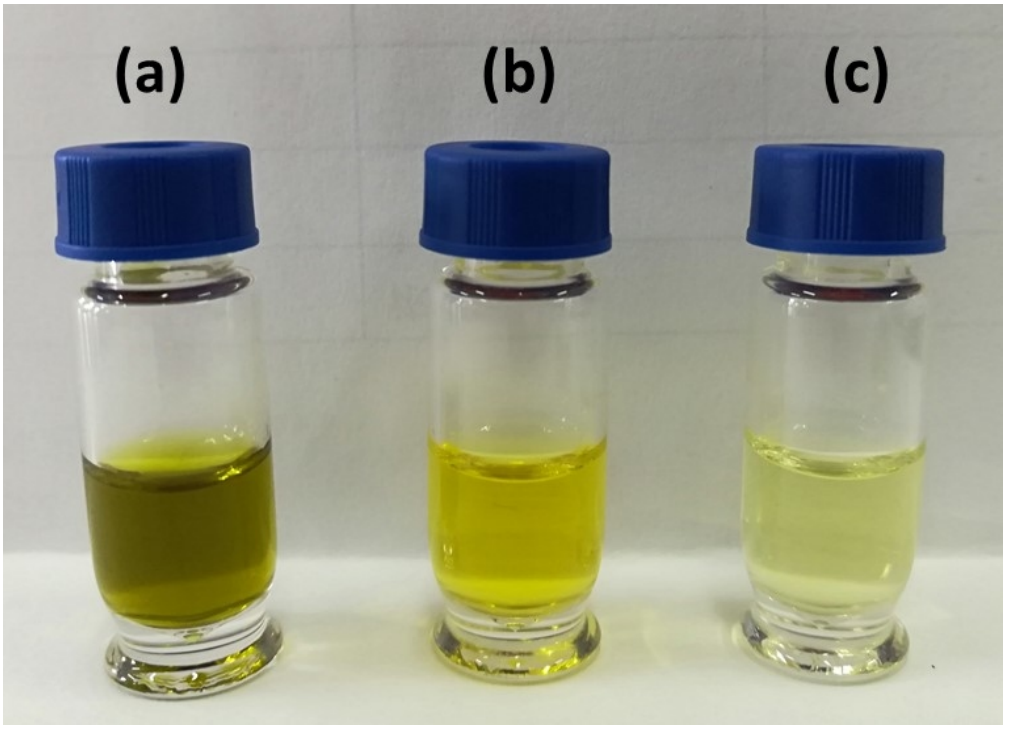

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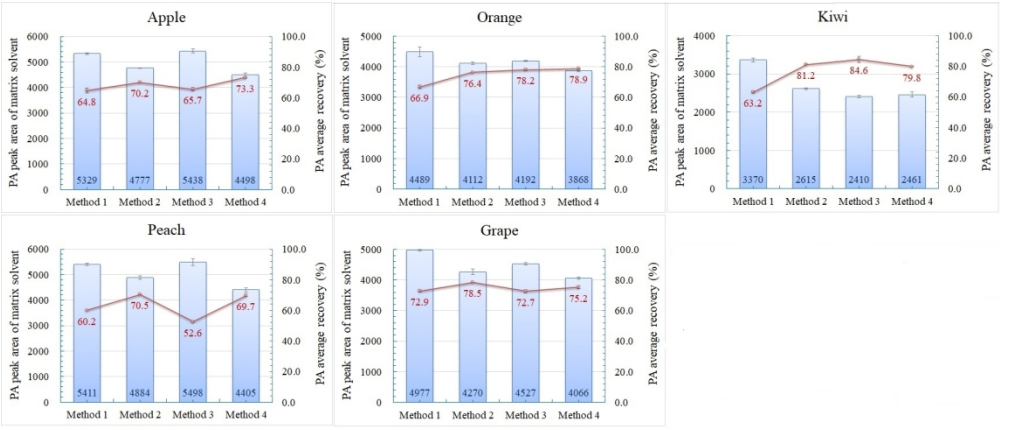

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