Soxhlet-assisted matrix solid phase dispersion to extract flavonoids from rape (Brassica campestris) bee pollen

Shuangqin Ma\textsuperscript{a,b,c}, Xijuan Tu\textsuperscript{a,b,c}, Jiagtao Dong\textsuperscript{a,b,c}, Peng Long\textsuperscript{a,b,c}, Wenchao Yang\textsuperscript{a,b,c}, Xiaqing Miao\textsuperscript{a,b,c}, Wenbin Chen\textsuperscript{a,b,c,*}, Zhenhong Wu\textsuperscript{a,b,c,*}

\textsuperscript{a} College of Bee Science, Fujian Agriculture and Forestry University, Fuzhou, PR China
\textsuperscript{b} MOE Engineering Research Center of Bee Products Processing and Application, Fujian Agriculture and Forestry University, Fuzhou, PR China
\textsuperscript{c} State and Local Joint Engineering Laboratory of Natural Biotoxin, Fujian Agriculture and Forestry University, Fuzhou, PR China

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\section*{A B S T R A C T}

Soxhlet-assisted matrix solid phase dispersion (SA-MSPD) method was developed to extract flavonoids from rape (Brassica campestris) bee pollen. Extraction parameters including the extraction solvent, the extraction time, and the solid support conditions were investigated and optimized. The best extraction yields were obtained using ethanol as the extraction solvent, silica gel as the solid support with 1:2 samples to solid support ratio, and the extraction time of one hour. Comparing with the conventional solvent extraction and Soxhlet method, our results show that SA-MSPD method is a more effective technique with clean-up ability. In the test of six different samples of rape bee pollen, the extracted content of flavonoids was close to 10 mg/g. The present work provided a simple and effective method for extracting flavonoids from rape bee pollen, and it could be applied in the studies of other kinds of bee pollen.

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1. Introduction

Extraction of interested compounds from matrix is the key issue in sample preparation. For solid, semi-solid, and viscous sample, matrix solid phase dispersion (MSPD) has been proved to be an effective extraction method [1]. In the classic MSPD procedure, samples are firstly blended with solid support materials. This blending operation exerts both shearing and grinding force to the samples. Then the mixed and disrupted materials are transferred into column for chromatography isolation, and suitable eluting solvent is selected to isolate the target compounds. As the extraction and clean-up step is accomplished simultaneously, MSPD offers distinct advantages of reduced solvent consumption, shortened extracting procedure, eliminated emulsion formation, and enhanced extraction efficiency [2].

Since the first introduction in 1989 [3], MSPD has become popular in a variety of environmental, food, and biological matrices [1]. Moreover, this method is still being evolved and improved. In recent years, studies on the modification of the classic MSPD have been reported. For example, the ultrasonic-assisted MSPD (UA-MSPD) was first reported by Ramos et al. [4], who used sonication to improve the contact between the matrix and the solid support. Compared with the classic MSPD, UA-MSPD showed higher extraction efficiency with decreased RSDs. This modified method has been applied to the extractions of antimicrobials [5], organochlorine pesticides [6], polycyclic aromatic hydrocarbons [7], and benzo[a]pyrene [8]. A modified method named vortex-assisted MSPD (VA-MSPD) [9–12] was directly extracting target materials in a centrifuge tube, and the elution step in MSPD was replaced by vortex, which makes the extraction simpler. This methodology has been applied in the extraction of pesticide residues and mercury species from fish tissues [10–12] and the aflatoxins from tigernuts [9]. Bead-beating assisted MSPD was reported to be an alternative method of VA-MSPD, and it was proved to have better recovery and quantification limits for the extraction of tebuconazole from frog tissues [13]. In addition, accelerated solvent extraction method was also reported to be able to assist MSPD extraction for pesticides [14]. Recently, continuous liquid-solid extraction (Soxhlet) assisted MSPD method was reported by Su et al. to extract pesticide contaminants from soil sample [15–17]. Literatures mentioned above show the trends of MSPD application; however, those studies are all concerned with the extraction of the potential hazardous substances.
and there is no report about using the modified MSPD method to extract natural functional compounds.

In the present work, a Soxhlet-assisted MSPD method was developed to extract flavonoids from bee pollen, which is a bee hive product regarded as nutritional supplement and functional food [18–22]. This is the first report of a modified MSPD applied for extracting flavonoids from bee pollen. Parameters of the described methodology were investigated and optimized. The extraction ability and cleanup utility were compared with the conventional solvent extraction and the classic Soxhlet method.

2. Materials and methods

2.1. Chemicals and materials

Commercially available rape (Brassica campestris) bee pollen was purchased from supermarkets in Fuzhou City. Each pollen sample was weighted as 250 g. Melissopalynological analysis was performed by using microscopy method [23], and the calculated purity of collected samples was all higher than 90%. C18 (40–60 mm, 60 A) was purchased from Sepax Technologies (Shanghái, China). Silica gel (200–300 mesh) was purchased from Aladdin (Shanghái, China). Neutral alumina (200–300 mesh), sea sand (0.65 mm–0.85 mm), rutin standard, ethanol, methanol, ethyl acetate, acetone, aluminum nitrate, potassium acetate, and anhydrous sodium sulfate were all purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghái, China).

2.2. Instruments

UV–Vis spectrophotometer (YOUKE-UV759CRT, Shanghai, China) was applied to the quantitative analysis of the target compounds. HPLC analyses were performed on Shimadzu 20AT equipped with SPD-M20A detector. A 5 μm WondaSil C18 column (250 × 4.6 mm) was used for chromatographic separation. 0.2% aqueous phosphoric acid solution (solvent A) and acetonitrile (solvent B) were used as the mobile phase. The gradient elution condition was as follows: 0 min, 5% B; 3 min, 6%; 10 min, 20%; 28 min, 26%; 33 min, 28%; 35 min, 65%; 45 min, 90%; 55 min, 90%; 60 min 80%; 65 min, 50%; 70 min, 5%. Flow rate was 1 mL/min.

Mobile phases were passed through a 0.22 μm membrane filter and degassed under vacuum condition. The column temperature was set at 35 °C, and the injection volume was 10 μL.

2.3. Soxhlet-assisted MSPD extraction

In the preliminary experiments, classic Soxhlet extractor was used to perform the Soxhlet-assisted MSPD (SA-MSPD). However, results showed that extracts could not pass through the siphon when the solvent reached the over-flow level. Inspired by the modified Soxhlet extractor [24], the commercially available constant pressure funnel was selected as the extractor in our method, in which the siphon is omitted, and the elution step can be simply controlled by the stopcock. The protocol of the methodology is illustrated in Fig. 1. Briefly, rape (B. campestris) bee pollen pellets were ground by pulverizer (LINDA-DFY300, Wenling, China), and then were delivered through 40 mesh sieves. Accurate 1.00 g sample and a certain amount of solid support were put into an agate mortar and ground for 2 min (Step 1). The obtained mixtures were transferred into a constant pressure funnel, in which degreasing cotton, anhydrous sodium sulfate (1.00 g), and additional dispersant (1.00 g) were preloaded (Step 2). A condenser tube was connected to the upper head of the constant pressure funnel, and the round flask which had 80 mL ethanol was connected to the bottom of the funnel. Extraction solvent was then heated and refluxed for extraction (Step 3).

2.4. Soxhlet extraction

Soxhlet extraction was performed in a classic Soxhlet extractor. Accurate 1.00 g sample was placed in an extraction bag filter and extracted with 80 mL ethanol.

2.5. Solvent extraction

Solvent extraction was performed based on the method reported by Moreno et al. [25]. Accurate 1.00 g sample was extracted with 80 mL ethanol with continuously stirring at room temperature for 8 h. The suspension was separated by centrifuging at 5000 rpm for 5 min.

2.6. Determination of total flavonoids content

Total flavonoids contents were analyzed using aluminum nitrate spectrophotometric method [26]. Resulting flavonoids extracts obtained by using the three different extraction methods were transferred into a 100 mL volumetric flask, respectively, and they were diluted to the volume by ethanol. Then 2 mL of the diluted solution was mixed with 0.5 mL aluminum nitrate solution (10%) and 0.5 mL potassium acetate solution (1 M) in a 25 mL volumetric flask, and then it was diluted to 25 mL with ethanol. After 10 min standing, the absorbance intensity at 402 nm was recorded by UV–vis spectrophotometer using the sample solution without coloration as the blank. Rutin was used for quantitation. Standard rutin solutions were prepared by mixing different volumes of rutin solutions (0.2 mg/mL) with 0.5 mL aluminum nitrate solution (10%) and 0.5 mL potassium acetate solution (1 M) in a 25 mL volumetric flasks and then diluting with ethanol to 25 mL. The volumes of rutin solutions used were from 1.0 mL to 3.0 mL with a 0.5 mL increment. The diluted solutions were set aside for 10 min before using. These standard solutions were detected at 402 nm, and the absorbance intensity and the concentrations were used to make the standard curve for quantification. Flavonoids content was expressed in mg of rutin/g of pollen.

3. Results and discussion

3.1. Extraction parameters of SA-MSPD

3.1.1. Extraction solvent

Four types of organic solvents including ethanol, methanol, acetone, and ethyl acetate were used to optimize the extraction. The extracted results were shown in Fig. 2a, which demonstrated that the extracted content of flavonoids decreased with the reduction of solvent polarity. The extracted flavonoids content obtained by using ethyl acetate was calculated to be only 5% of that extracted by ethanol. SA-MSPD showed more significant difference between the extraction efficiencies of ethyl acetate and alcohol than the results reported using liquid extraction [27]. This might result from the separation effect of the column chromatography in MSPD method. Such difference also suggested that interference components can be eliminated by using suitable extraction solvent, and that the types of sorbent solid support and extraction solvent can be optimized to isolate different target compounds in SA-MSPD preparation. The statistical analysis indicated that there was no significant difference between ethanol and methanol (P > 0.05). As ethanol is less expensive and more environmentally-friendly than methanol, ethanol was used as the extraction solvent in the following studies.
Fig. 1. Operation procedure of SA-MSPD method. Step 1: the sample is blended with solid support. Step 2: the blended materials are transferred into the column of commercial constant pressure funnel, and then compressed. Step 3: continuous elution with suitable solvent is performed with the aid of condensation and heating device.

Fig. 2. (a) The extraction abilities of four extraction solvents in SA-MSPD (methanol, ethanol, acetone, and ethyl acetate), Soxhlet after 1 h and 7 h extraction, and solvent extraction after 8 h. SA-MSPD extraction: silica gel as solid support with mass ratio of samples to solid support of 1:2, extraction solvent of 80 mL ethanol, and extraction time of 1 h. Soxhlet extraction: extraction solvent of 80 mL ethanol and extraction time of 1 h and 7 h. Solvent extraction: extraction solvent of 80 mL ethanol and extraction time of 8 h. (b) Variation of extracted flavonoids content with increasing extraction time in SA-MSPD. SA-MSPD extraction: silica gel as solid support with mass ratio of samples to solid support of 1:2, and extraction solvent of 80 mL ethanol.
3.1.2. Extraction time

In the classic MSPD procedure, the volume of the elution solvent plays an important part in extraction yields of target compounds. In SA-MSPD method, as the solvent is continuous refluxing and flowing through the MSPD column, the total volume of extraction solvent is nearly unchanged. However, the extraction yield can be affected by the extraction time. The extracted content of flavonoids with different extraction time were shown in Fig. 2b. It showed that content of extracted flavonoids increased with the extension of extraction time and reached plateau after 1 h. This extraction time was much shorter than the published required time, 5 h–48 h, for the extraction of the flavonoids from bee pollen [19,21,22].

In order to evaluate and compare the extraction ability of SA-MSPD, conventional solvent extraction and Soxhlet extraction were added into the study. The results were shown as the additional bars in Fig. 2a. The extracted flavonoids content of 1 h extraction was much higher using SA-MSPD than Soxhlet method, but it was notable that the flavonoids reach plateau at 7 h (Fig. S1) using Soxhlet method. The highest extraction yield of Soxhlet method showed no significant difference with that of SA-MSPD. The result indicated that SA-MSPD method needs less extraction time to reach the highest yield of Soxhlet method. Comparing with conventional solvent extraction, SA-MSPD method showed higher extraction efficiency. As shown in Fig. S2, solvent extraction, accomplished at 8 h, extracted approximately 70% flavonoids content of that obtained by SA-MSPD. Similar extraction efficiency difference has been observed in other matrices in comparisons of classic MSPD with the conventional methods [28,29]. The difference could be attributed to the disruption and dispersion effects of solid supports, and it could also result from the surface area enhancements for the subsequent extraction of the samples [30].

SA-MSPD also showed advantages of low consumption of labor and organic solvent. As the fresh extraction solvent is continuous condensed and eluted in SA-MSPD method, which allows the elimination of the repeated extraction step that is normally required in traditional solid–liquid extraction to guarantee extraction yields [20,22].

3.1.3. Solid supports

Three types of sorbents, C_{18}, silica gel, and neutral alumina, with four different mass ratios of samples to sorbents were tested to investigate the effect of the solid support condition to the yields (Fig. 3). The highest extraction yields were achieved at the mass ratio of 1:2 for both C_{18} and silica gel. Slight decreases in extraction yields were observed when the ratio is increased to higher than 1:2. It could result from the increase of the sorbents leading to stronger retention to flavonoids. Neutral alumina resulted lower extracted content than C_{18} and silica gel. Alumina may have strong adsorption effect toward flavonoids [31], and the elution condition in our method cannot fully elute the adsorbed flavonoids. Therefore, silica gel was selected as the solid support because of higher yields and lower cost in the three tested sorbents, and the mass ratio was set at 1:2.

A non-retentive solid support, sea sand, was used in SA-MSPD procedure to investigate the sorbent function. As shown in Fig. 3, the extracted content is lower than C_{18} and silica gel. The micrometer scales of C_{18} and silica gel materials provide larger surface areas for the disruption of samples, which would be helpful for the contact of samples with extraction solvent, and result in the better extraction yields.

The obtained extracts were analyzed with HPLC-DAD to estimate the clean-up effect of SA-MSPD. In HPLC analysis, gradient elution of acetonitrile and 0.2% aqueous phosphoric acid (v/v) on a reversed-phase C_{18} column were performed to separate the extracted compounds. The full HPLC chromatogram of the extracts using sea sand as solid support was shown in Fig. S3. Compounds in the range between RT 3–4 min and 43–50 min are the solvent contamination, thus the chromatography in the range of RT 4–43 min was studied to investigate the clean-up ability. The typical chromatograms were shown in Fig. 4. Components in the chromatogram profiles of three extracts, sea sand, Soxhlet method, and solvent extraction, were nearly the same, which suggests that sea sand would not have clean-up ability toward bee pollen sample. However, the peak areas of all components obtained by solvent extraction were lower than those of sea sand and Soxhlet. This result was consistent with the result obtained in spectrophotometric study. The chromatogram of the extract using silica gel as solid support was shown in Fig. 4d. The result could be the evident that silica gel exhibits cleaner chromatogram comparing with sea sand. At the short retention time, the peak of high polar interference compound (Peak No. 1) was eliminated, and it may result from the adsorption ability of the polar surface of silica gel. Moreover, significant clean-up effects were observed at Peak No. 5 and 14, of which peak areas significantly reduced. The result suggested that the present method also has clean-up effects toward lower polarity interference from bee pollen. The interested clean-up effect may be beyond the supposition of silica gel sorbent. Baker [32] suggested that since the sample matrix become part of the chromatographic phase in MSPD, the matrix interacts with the sorbents and the mobile phase would make the elution of target analytes become not readily predictable. The outer wall of our sample matrix, pollen grains, is sporopollenin, which is a natural polymer composed of hydrophobic aromatic unsaturations and hydrophilic carboxylic acids, ethers, and hydroxyls [33]. Recently, sporopollenin has been used as a novel sorbent for separation studies [34,35], and the pollen grain also has been used as solid-phase extraction sorbent [36]. It is reasonable to speculate that the matrix interaction of bee pollen would take part in separation action, and that combination of hydrophobic and hydrophilic interaction would result in clean-up effect. The 14 peaks were not further identified because flavonoids generally occur in plants as glycosylated derivatives, while commercial standards are provided in the form of aglycones in most case [37]. However, the above results indicated that SA-MSPD procedure exhibits clean-up effects without reducing extraction yields.
3.2. Application in real samples

Six different rape (Brassica campestris) bee pollen samples obtained from supermarkets were analyzed using the developed SA-MSPD and the conventional solvent extraction method. As shown in Table 1, extracted flavonoids content was between 9.69 and 10.90 mg/g by SA-MSPD, while the content of flavonoids extracted by solvent extraction was between 6.40 and 7.88 mg/g. The higher extraction yields of SA-MSPD would attribute to the improved extraction efficiency which has been discussed above.

4. Conclusions

In summary, a modified SA-MSPD method was developed for extracting flavonoids from bee pollen samples. This optimized method offered yields of approximately 1% sample weight, which is similar to that obtained by Soxhlet method and higher than that obtained by using the conventional solvent extraction method. HPLC studies demonstrated the clean-up effects of SA-MSPD. Comparing with conventional method, SA-MSPD requires less labor, shorter extraction time, and less solvent. SA-MSPD would be a feasible method for extracting and cleaning up flavonoids from bee pollen sample with advantages of simple preparation and high efficiency.

Conflict of interest

The authors have declared no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2015.09.038.

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