Application of solid-phase microextraction for determination of flavonoids from plant foods

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Abstract

The analysis of flavonoids requires tedious extractions and derivatization before GC determination. A simple and efficient technique that does not require solvent and uses less operating time for the determination of the flavonoids, found in fruits and vegetables, by utilizing solid-phase microextraction (SPME) followed by on-fiber derivatization technique has been developed. Two flavonoids, quercetin and myricetin, were used to investigate the scope and applicability of our method. Compounds in water with 10% sodium chloride were first headspace extracted on a 85 μm polyacrylate (PA)-coated fiber at 55 °C for 30 min, followed by on-fiber silylation in a vial containing 20 μL of bis(trimethylsilyl)trifluoroacetamide (BSTFA) at 45 °C for 10 min, and then desorbed at 280 °C for 3 min. The identification of the flavonoids was performed by GC-MS.

Keywords：solid-phase microextraction, flavonoids, GC-MS, fruits, vegetables

1. Introduction

Flavonoids are a class of polyphenolic compounds widely distributed in plants and as part of the human diet[1]. These compounds have received particular attention in recent years. The epidemiological studies have been shown the biological effects of flavonoids in role of health promotion, including free radical scavenging (antioxidant activity)[2-5], and coronary heart disease preventing[6,7]. Dimitrios had provided an overview of the findings related to the presence of antioxidant phenols in plant sources[8]. Flovonoids are categorized as flavonol, flavanol, flavanone, flavone, anthocyanidin and isoflavone. Among these compounds, flavonol

(e.g. quercetin, myricetin and kaempferol) are the major flavonoids found in vegetables and fruits[9,10]. In edible parts of plants flavonols occur as aglycones and glycosides, in which sugar molecular is bound to various positions in the phenolic groups, so the hydrolysis procedure is needed for the determination of flavonol aglycones.

Various analytical methods have been proposed for the separation and determination of these biologically active phenolic components in food. Most of these protocols are based on high-performance liquid chromatography (HPLC) techniques, coupled with UV spectrophotometry, electrochemical detection (ED) methods, or mass spectrometry[11]. Merken *et al*. developed a HPLC system for the separation and quantification of seventeen flavonoids in commonly consumed foods[12]. Brodbelt and coworker reported an improved method for identifying the predominant flavonols, quercetin and kaempferol of kale by high-performance liquid chromatography electrospray ionization mass spectrometry (HPLC-ESI-MS)[13]. Biesaga and colleagues developed a HPLC separation method with diode array detection for quantitative determination of quercetin in some food (onion, apple) and herbal product[14]. Häkkinen and Auriola disclosed a HPLC-ESI-MS method for the analysis of flavonol aglycones and glycosides in berries, and used the photodiode-array detection (DAD) for identification of aglysones[15]. Escarpa *et al*. determined the phenolic compounds from different apple varieties using HPLC-DAD method[16]. Other technique such as capillary zone electrophoresis (CZE)[17], capillary electrochromatography (CEC) have been employed to study the flavonoids in plants[18].

Other than liquid chromatography, gas chromatography (GC) provided an alternative method for the determination of flavonoids in food sample. GC methods provide high resolution and low detection limits, however, flavonoids have high melting point, and decompose when heated above their melting point. Consequently, trimethylsilyl (TMS) derivatives of flavonoids are prepared for GC analysis to increase the volatility and to improve the thermal stability of the flavonoids. Komatis and colleagues utilized a GC-MS method for characterization of flavonoids and phenolic acids in aromatic plants[19,20]. Tokuşoğlu *et al*. determined the amount of flavonols in tomato-base products by RPHPLC and confirmed the presence of the flavonol aglycons by GC-MS [21].

Most of these methods require traditional solvent extraction or a solid phase extraction (SPE)[22] clean-up procedure prior to HPLC or GC analysis. However, the extractions often demand laborious works and large amount of organic solvent consumption. The solid phase microextraction (SPME), developed by Pawliszyn and co-woekers[23,24], is a viable alternative to solvent extraction and offers a convenient, solvent-free and time-saving method which has

been widely used[25-30]. Applications in food analysis, the SPME methods are mostly used for the investigation of various flavor composition[31-34], pesticides[35-37] and herbicides[38] in food samples[39]. For the analysis of fresh vegetables and fruits, López *et al*. evaluated the volatile profiles of avocado purees using SPME and GC-MS after microwave processing[40]. Melton and colleagues applied the headspace SPME to analyze volatile flavor profile development during storage and ripening of kiwifruit[41]. Nazimah and coworker analyzed the volatile compounds from Malaysian durians (*Durio zibethinus*) using HS-SPME coupled to fast GC-MS[42]. McFeeters *et al*. reported a SPME technique for measuring the generation of fresh cucumber flavor compounds[43]. Andrade and colleagues developed a HS-SPME/GC-MS method to analyze the VOCs in red, yellow and purple varieties of *Capsicum chinense* sp. Peppers[44]. Hu *et al*. reported a HS-SPME/GC-MS technique to investigate the changes in the volatile compounds and chemical and physical properties of Yali pear (*Pyrus bertschneideri* Reld) during storage[45]. The aroma compounds of edible herb[33,46,47] and green tea[48] were also verified using the SPME/GC methodology.

The result of GC analysis for low-volatility polar compounds exhibits low sensitivity and tailing. To increase the recovery, SPME has been coupled with derivatization processes [28,49-51]. Compared with direct SPME, the coupling of derivatization with SPME during sampling affords an improvement in selectivity and sensitivity of analysis[52]. A new method has been implemented for derivatization on solid phases to alleviate the problems posed by interferences and the extra steps associated with classical derivatization[50]. Pawliszyn *et al*. analyzed the anatoxin-a in aqueous samples by solid-phase microextraction coupled to HPLC with fluorescence detection and on-fiber derivatization by dropping or spraying the fluorogenic derivatizing reagent onto the fiber containing extacted analytes[53]. Campins-Falco *et al*. reported the analysis of methylamine by SPME and HPLC after on-fibre derivatization with 9-fluorenylmethyl chloroformate[54]. Tsai and Chang applied SPME with on-fiber derivatization to analyze aldehydes in water by HS-SPME extraction of the aldehydes in water sample with *O*-2,3,4,5,6-(pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA) loaded fiber, followed by GC-MS analyses of oximes formed[55]. Rodriguez *et al*. employed SPME followed by on-fiber silylation to determine the anti-inflammatory drugs in water samples using *N*-methyl-*N*-(tert-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA) as the derivatizing reagent[56]. Lee *et al*. reported the application of on-fiber silylation for the determination of leaching of bisphenol A from plastic containers by SPME and GC-MS[57].

To the best of our knowledge, there was only one SPME method for the determination of isoflavone aglycones gentistein and daidzein in urine in literature[58]. Brodbelt and coworkers

employed solid-phase microextraction–high-performance liquid chromatography–electrospray ionization mass spectrometry (SPME–HPLC–ESIMS) in these analyses. However, the analysis of the flavonoids in fresh vegetables and fruits by SPME coupled with GC-MS has yet to be established because significant reduction of sampling time and high sensitivity for minor components in complex matrix can be achieved.

Additionally, The epidemiologic literature has reported that consumption of higher levels of vegetables and fruit could reduce risk of cancer at most sites, and particularly with epithelial cancers of the alimentary and respiratory tracts[59]. The rheumatoid patients subjectively benefited from the vegan diet rich in antioxidants, lactobacilli and fibre [60]. The vegetarian diets are now consumed in common all over the world. There are many varieties of vegetable and fruit in Taiwan, however, the identification of their flavonoids contents is yet to be studied. Consequently, there is an urgent need for a quick and reliable method for analyzing these plant foods to establish their flavonoid profile, as a starting point for its commercial exploitation of these high quality Taiwan agricultural products. Opening up of the domestic and foreign markets will benefit the Taiwan's fruit and plant economy.

2. Experimental

2.1. Materials

Quercetin (Qu), myricetin (My) and the derivatization agent BSTFA (bis(trimethylsilyl)trifluoroacetamide) were purchased from Aldrich (Steinheim, Germany). HPLC-grade methanol was purchased from Merck (Darmstadt, Germany). Sodium chloride was supplied by Panreac (Barcelona, Spain). A manual SPME fiber holder and two types of SPME fibers, polyacrylate (PA, 85 μm) and polydimethylsiloxane/ divinylbenzene (PDMS/DVB, 65 μm) were purchase from Supelco (Bellefonte, PA, USA).

2.2. Analytical procedure

2.2.1. Solution preparation

Standard stock solutions (1000 μg mL^{-1}) of each flavonol were prepared in methanol and were stored in a refrigerator. Working solutions were prepared by mixing each of the standard stock solutions with ultra pure water, and prepared freshly every three days.

2.2.2. SPME

A 3.0 mL sample solution in a 4 mL vial with sodium chloride (0.1 g mL^{-1}) was extracted by direct immersion of SPME fiber using a magnetic stirrer provided constant agitation during extraction. The extraction was carried out in a 55 °C water bath for 30 min.. After the extraction is completed, the fiber was dipped into ultra pure water with stirring for 20 sec. Then any trace of water on the fiber was wiped out by a soft tissue.

2.2.2. On-fiber silylation

Following the extraction step, the SPME needle was pierced through the Teflon-backed silicone septum into the headspace of a 2 mL vial containing 20 μL of BSTFA with a magnetic stir bar at 45 °C (water bath), and the fiber was exposed in the headspace for derivatization for 10 min. After derivatization, the SPME fiber was inserted into the GC injection port to achieve the thermal desorption.

2.3. GC/MS parameters

Gas chromatography-mass spectrometry was performed on a Hewlett-Packard 6890 gas chromatograph interfaced with a HP 5973 mass-selective detector (using 70ev electron ionization). Mass spectra of the derivatives were obtained in the range of *m/z* 50 ~ 750. Gas chromatographic separation was conducted using a DB-5MS capillary column (30 m x 0.25 mm i.d., 0.25 μm film thickness) in splitless injection mode. Carrier gas was He (purity 99.995%) at 1.0 mL/min flow rate. The initial oven temperature was 100 °C, held for 1 min, the temperature was raised to 280 °C at a rate of 10 °C/min, held for 5 min, then temperature was raised to 300 °C at a rate of 20 °C/min, held for 8 min, the total elution time was 32 min. The injection-port was set to 280 °C. For SPME analysis a Supleco 0.75 mm i.d. GC inlet liner was used.

3. Results and Discussion

3.1. Microextraction conditions

Two major flavonols found in vegetables and fruits, quercetin and myricetin were used in the model study. Several variables have been examined to determine their roles in extracting phenolic components: the duration of extraction, addition of salt, and the adsorption capability of different fibers, such as polyacrylate (PA) fiber and polydimethylsiloxane-divinylbenzene (PDMS-DVB).

3.1.1. Heating temperature and extraction time

The extraction effect is depended on the transport of the analytes to the fiber. For semivolatile compounds the increase in temperature causes acceleration of the moving of analytes to the SPME coating, consequently enhances the sensitivity. For optimizing the temperature effect on extraction of the objective compounds, the vials containing aqueous sample solution were heated at three different temperatures (45, 50, and 55°C), shown in Fig 1. Higher responses were observed for quercetin studied at 55 °C, therefore, 55 °C was selected for our study.

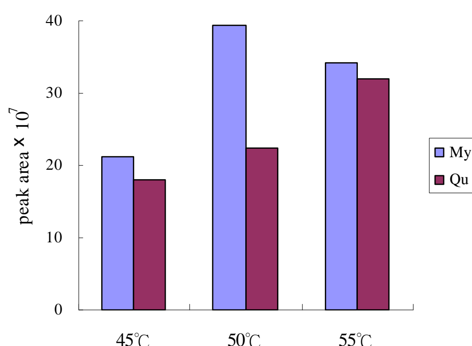

Fig. 1. Effect of extraction temperature using a PA fiber.

The results of peak area versus extraction time up to 50 minutes on a standard solution of concentration of 10 μg mL^{-1} for each of the flavonols were illustrated in Fig. 2. The adsorbed amount of the flavonols reached to a maximum at 30 min and started to decrease afterwards for both the flavonols. Therefore, 30 min was chosen as the optimum extraction time.

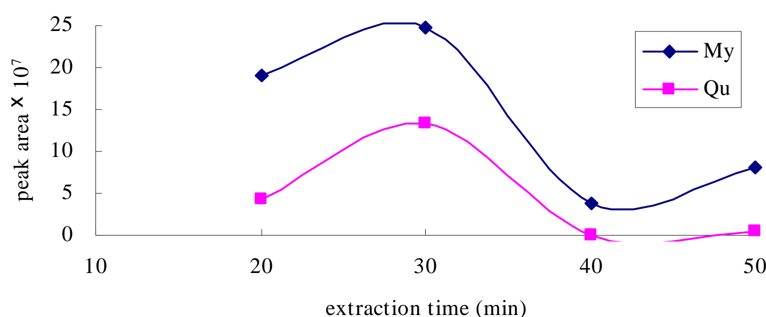

Fig. 2. Extraction time profiles at 55 °C. Samples with NaCl added ((0.1 g mL^{-1}).

3.1.2 Addition of sodium chloride

Because the addition of salt could produce a significant salting out effect which will decrease the solubility of the analytes in the solution and will result in a considerable

improvement in sensitivity, we have studied the effect using different amount of salt. As shown in Fig. 3, the highest signal intensity for quercetin and good result for myricetin were obtained when 10% sodium chloride was used. We decided to use 10% NaCl as the salt in our experiments consequently. To reduce the interference of further derivatization with salt on the surface of fiber, the fiber was exposed in pure water for 20 sec after extraction.

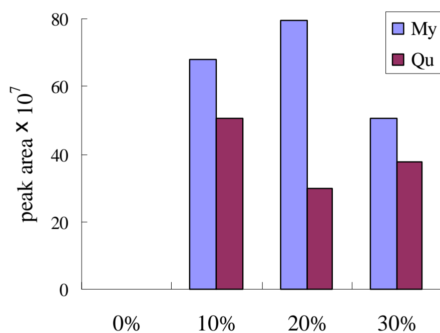

Fig.3. Effects of addition of salts on peak areas.

3.1.3. Selection of SPME coating

Two different fibers were evaluated using the optimal sampling conditions (Section *3.1.1 and 3.1.2*) to determine which fiber most effectively extracted flavonol compounds from sample solutions. The results of the fiber screening were shown in Fig. 4. The adsorption of analyte to fiber coating is dependent on the chemical nature of the target analytes (polarity and volatility). Higher extraction efficiency was obtained by the relatively polar PA coating for the polar flavonol compounds. However, under this condition the PDMS/DVB fiber had an extremely low sorption capacity. Hence the PA fiber was selected for further studies.

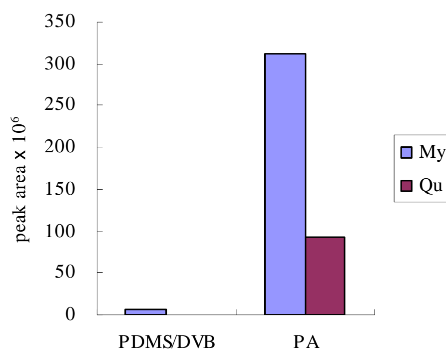

Fig. 4. Comparison of extraction efficiency obtained with two different fibers.

3.2. On-fiber derivatization conditions

In general, three different approaches have been utilized for the derivatization on solid

phases [61], namely, in the sample matrix, on the fiber (after sampling), and in the GC injection port.

Since direct immersion of the fiber in the organic derivatizing agents will cause the spoilage of the coating on the fiber, on-fiber headspace solid-phase microextraction derivatization procedures have been explored in order to prolong the life of the fiber. Furthermore, the moisture sensitive derivatization reagents can be used simply after extraction of analytes from aqueous sample solutions. The derivatization time and temperature have been examined to determine their roles in converting the flavonol components into more volatile analytes.

Since the BSTFA will damage the fiber coating at elevated silylation temperature e.g. above 60°C, the derivatization conditions were evaluated at 45°C. The time effect of BSTFA derivatization was examined. As seen in Fig. 5, the reaction was carried out at 45°C for 10 to 20 min, the area counts of the TMS derivatives of quercetin and myricetin reached the highest sensitivity at 10 min. Hence, the derivatization conditions of 45°C and 10 min were selected for later studies.

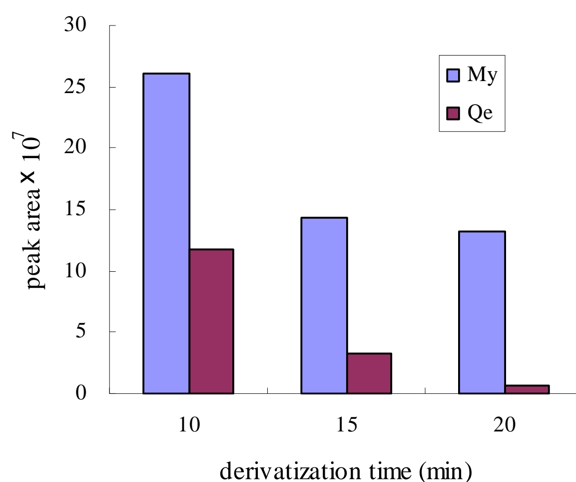

Fig. 5. Derivatization time profile at 45°C.

3.4. Performance of the GCMS

The two mass spectra of TMS derivative of flavonols were shown in Fig. 6. The molecular ion $[M]^+$ of TMS derivatives in all of the mass spectra was vanished. The elimination of a TMS moiety and a methyl radical $[M\text{-}TMS\text{-}Me]^+$ for quercetin was the most dominant one making it the base peak, while the base peak of myricetin was $[M\text{-}3TMS\text{-}3Me]^+$ ion. The other characteristic main fragment ions in this assay are listed in Table 1.

Table 1. Fragmentation ions of the TMS derivatives of flavonols.

Compound	$[M]^+$, *m/z*	Characteristic ions, *m/z*
Quercetin Qu	662	647, 575, 487
Myricetin My	750	415, 487, 559

Underlined ions were base peak.

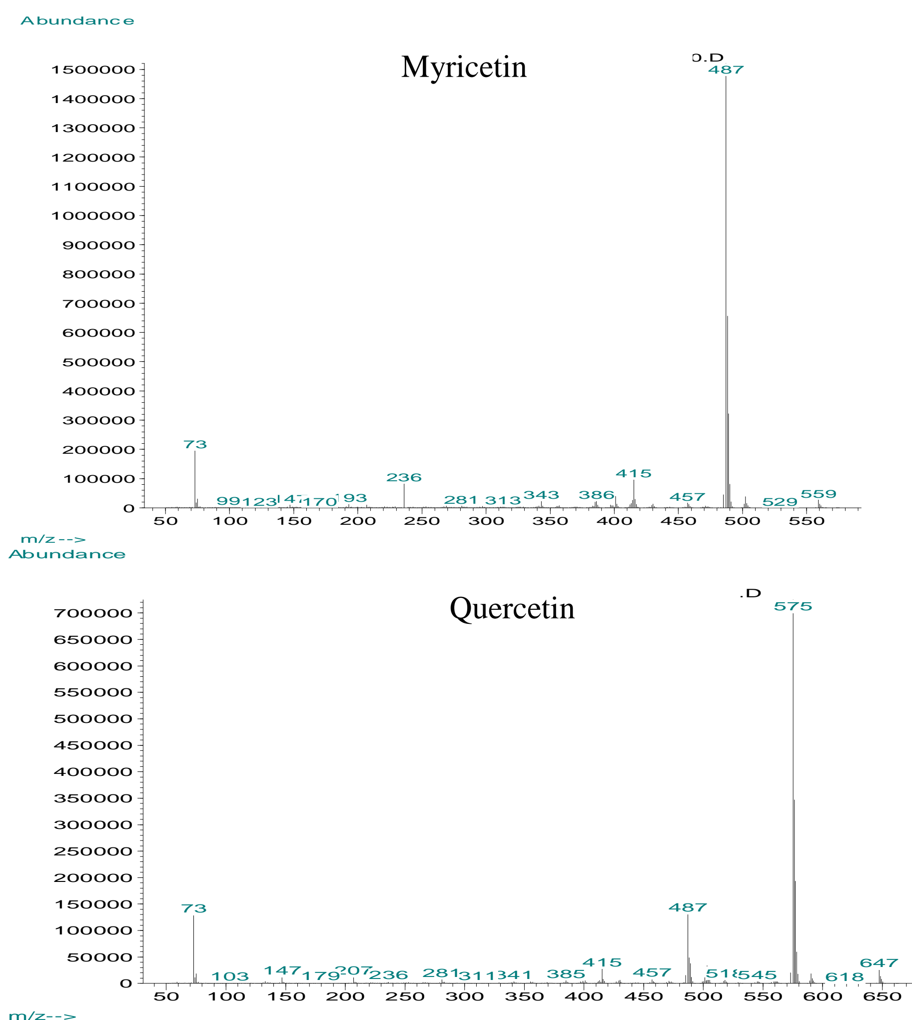

Fig. 6. Mass spectra (in scan mode) of TMS derivative of flavonols

4. Conclusions

A simple, efficient, and highly selective method for the rapid determination of the flavonoids utilizing SPME and on-fiber derivatization procedure have been established.

Due to savings of the analysis time, elimination of solvent, and the avoidance of possible contaminants from sample matrix, this method we explored will find its applications not only in academic laboratories, but also will be a very useful and practical method in the evaluation of the nutritional value of edible plants.

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