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An automatic on-line 2,2-diphenyl-1-picrylhydrazyl-high performance liquid chromatography method for high-throughput screening of antioxidants from natural products

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ABSTRACT

Many natural products are rich in antioxidants which play an important role in preventing or postponing a variety of diseases, such as cardiovascular and inflammatory disease, diabetes as well as breast cancer. In this paper, an automatic on-line 2,2-diphenyl-1-picrylhydrazyl-high performance liquid chromatography (DPPH-HPLC) method was established for antioxidants screening with nine standards including organic acids (4-hydroxyphenylacetic acid, *p*-coumaric acid, ferulic acid, and benzoic acid), alkaloids (coptisine and berberine), and flavonoids (quercitrin, astragalin, and quercetin). The optimal concentration of DPPH was determined, and six potential antioxidants including 4-hydroxyphenylacetic acid, *p*-coumaric acid, ferulic acid, quercitrin, astragalin, and quercetin, and three non-antioxidants including benzoic acid, coptisine, and berberine, were successfully screened out and validated by conventional DPPH radical scavenging activity assay. The established method has been applied to the crude samples of *Saccharum officinarum* rinds, *Coptis chinensis* powders, and *Malus pumila* leaves, consecutively. Two potential antioxidant compounds from *Saccharum officinarum* rinds and five potential antioxidant compounds from *Malus pumila* eaves were rapidly screened out. Then these seven potential antioxidants were purified and identified as *p*-coumaric acid, ferulic acid, phloridzin, isoquercitrin, quercetin-3-xyloside, quercetin-3-arabinoside, and quercetin-3-rhamnoside using countercurrent chromatography combined with mass spectrometry and their antioxidant activities were further evaluated by conventional DPPH radical scavenging assay. The activity result was in accordance with that of the established method. This established method is cheap and automatic, and could be used as an efficient tool for high-throughput antioxidant screening from various complex natural products.

1. Introduction

Antioxidants play a crucial role in preventing or postponing a variety of diseases, such as cardiovascular and inflammatory disease [1], diabetes [2] and breast cancer [3], because of their ability to capture or repair damages caused by active oxygen species [4,5]. Antioxidants include natural antioxidants [6], such as ascorbic acid, and synthetic antioxidants [7], such as 2,6-di-*tert*-butyl-4-methylphenol. Synthetic antioxidants have been widely used in the food industry for extending the shelf life of foods. However, there are some arguments about their safety and adverse effects [8,9]. So in the past decades, numerous researchers have focused on natural antioxidants that are more diverse in structure and bioactivity and less toxic, compared with the synthetic ones [10,11]. Currently, many natural products are good candidates of natural antioxidants [12–14]. Searching antioxidants from natural products has been paid to attention because of safety and high-value. Unfortunately, the great complexity of natural products is a major obstacle for this kind of research.

In order to discover new natural antioxidants, many antioxidant activity assays have been developed [15]. Among these assays, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity assay, which was discovered and developed by Blois [16] and Brand-Williams [17], has been widely used for its easy operation and stable reaction system. This traditional method was commonly done by pharmacological and clinical active tracking of different solvent fractions of natural products using spectrophotometry method of the colorimetric estimation of DPPH. The antioxidant capacity was usually expressed in gallic acid equivalents, Trolox equivalents, DPPH IC_{50} (the effective concentrations at which the

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initial DPPH radicals were scavenged by 50%), and so on. Then every compound from the fractions with antioxidant activity was purified, and the antioxidant activity of each purified compound was further detected by DPPH radical scavenging activity assay once more [18–25]. However, this assay method is not applicable to colored plants because of the interference by pigments. The procedure of this traditional method is time-consuming, arduous, and less efficient. Though antioxidant assay can determine antioxidant activity of single compound or natural product extracts, it cannot determine every compound from the extract of natural product. In order to overcome the above-mentioned problems, it is essential to establish a simple, rapid, and effective method for screening and purifying potential antioxidants from complex natural products. A large number of studies have been published over the past decades on the combination of spectrophotometry DPPH radical scavenging assay with either a high performance liquid chromatography (HPLC) or thin layer chromatography (TLC) separation [26–29]. The TLC method is simple, rapid, cheap, and flexible. However, the separation ability of this method was much lower than that of HPLC because several compounds might be co-eluted as a single spot in TLC [30]. In 1998, Yamaquchi et al. [31] used a HPLC method for evaluation of the DPPH radical scavenging activity. Then Tang et al. [30] developed a simple and time-saving off-line DPPH-HPLC method. This method included the reaction of DPPH with crude extracts first, and then HPLC separation analysis. By comparison of the peak area change, it could be known which compounds were responsible for the overall antioxidant activity. In this method, DPPH solution and natural products could mix adequately to get a complete reaction. So through only one time reaction, all potential antioxidants could be detected in a short time. As in many previously reported chromatographic methods, this method was expected to be useful for determination in colored samples because the interference of pigments could be avoided through separation of HPLC. It could screen out directly antioxidants from natural products [32–34] and avoid the time-consuming isolation of individual compounds. Unfortunately, this off-line DPPH-HPLC method could be completed just manually, and was not suitable for high-throughput analysis.

The automation of parameter optimization and operation of sample analysis is of great interest for the large number of samples due to its time-saving, especially when a high- throughput is required to process samples over 24 h. Therefore, an on-line HPLC-DPPH method has been utilized for the detection of antioxidants from complex mixtures by on-line post-column reaction of eluates with free radicals and has resulted in the identification of several antioxidant compounds [35–37]. During this procedure, the reaction time was set shortly (most <1 min), which might cause false-negative results for antioxidants with slow kinetics of reactions. Meanwhile, this method needs much more amount of DPPH solution, and the reaction might not be complete and continuous in reaction coil compared with off-line DPPH-HPLC method. Some antioxidant active ingredients might not be screened out when this method is used for natural antioxidant screening. Therefore, an automatic method that can screen antioxidants fast, completely and efficiently has become a very important matter for natural antioxidant screening.

Up to now, in spite of the fact that many methods have been reported on screening and identifying antioxidants from natural products, those reported references mostly described the method for the one or two classes of compounds [38–40]. For example, Tang et al. [30] screened antioxidants phenolic acids from Chinese herbal *Lonicerae japonicae* flowers using an off-line DPPH-HPLC-DAD-TOF/MS method. Geng et al. [41] screened antioxidants flavonoids from *Limonium aureum* using an on-line HPLC-DPPH assay. Agatonovic-Kustrin et al. [26] simultaneously determined the content and antioxidant activity of apigenin, chamazulene and bisabolol from leaf and flower head extracts of feverfew, German chamomile and marigold using a HPTLC-DPPH method. However, antioxidants are diverse rather than one or two classes in natural products. Some antioxidants might be leaked out during the screening process with these previously reported methods. So, it is necessary to establish a method suitable for screening a wide range of natural antioxidants including organic acids, alkaloids and flavonoids.

Based on the above background about screening antioxidants from natural products, the aim of the present study was to establish an automatic on-line DPPH-HPLC method with nine standards, representing different classes of compounds organic acids, alkaloids, and flavonoids. The method overcame disadvantages of the methods off-line DPPH-HPLC and on-line HPLC-DPPH and was applied for high-throughput automatic screening of antioxidants from *Saccharum officinarum* rinds, *Coptis chinensis* powders, and *Malus pumila* leaves. This established method affords a new technique for efficient and high-throughput screening antioxidants from natural products.

2. Experimental

2.1. Standards, reagents and materials

Standards including 4-hydroxyphenylacetic acid (Compound 1), *p*-coumaric acid (Compound 2), ferulic acid (Compound 3), benzoic acid (Compound 4), coptisine (Compound 5), quercitrin (Compound 6), berberine (Compound 8), and quercetin (Compound 9) were purchased from Sigma-Aldrich (Shanghai, China). Astragalin (Compound 7) was isolated by counter-current chromatography (CCC) in our laboratory. The purity of all these standards was more than 98%. HPLC grade methanol was obtained from Fisher Scientific (Fair Lawn, NJ, USA). HPLC grade trifluoroacetic acid (TFA) and DPPH were purchased from J&K Chemical (Beijing, China). Ultrapure water used throughout the whole study was obtained by a Synergy Purification System (Millipore, Molsheim, France). All other reagents or solvents were of analytical grade. All the solutions were filtered through 0.45 μm PTFE membrane filters and degassed by ultrasonic bath before use.

Saccharum officinarum rinds were purchased from the market in Beijing city. *Coptis chinensis* powders were purchased from Bobai, Guangxi province. *Malus pumila* leaves used as raw material in this study were collected from apple production base in Yantai, Shandong province, China.

2.2. Preparation of DPPH, standards and crude extracts

Fresh DPPH solution of 5.0 mg/mL was prepared by dissolving 50.0 mg of DPPH in 10 mL of pure methanol on each day of analysis and diluted to low concentrations (0.1, 1.0, 2.0 mg/mL) with methanol, in a volumetric flask protected from light.

A mixed standard solution of 200 μg/mL was prepared by dissolving 2.00 mg of each individual reference compound in pure methanol and diluting to a final volume of 10.0 mL. To ensure the complete dissolution, ultrasonication was used for 20 min. A series of working calibrator solutions (10 μg/mL–200 μg/mL) of mixed standard solution were prepared by dilution of the mixed standard solution with pure methanol. All the mixed standard solutions were stored in brown flasks at 4 °C.

In order to prepare material for the forthcoming step, *Saccharum officinarum* rinds were dried with oven at 45 °C, and then ground into powder by a crusher. Firstly, 10 g of the powder was dissolved into 100 mL of 2 mol/L NaOH solution and stirred for 4 h at room temperature. Then 2 mol/L HCl was used to adjust the solution to pH 2. Finally the aqueous solution was extracted with ethyl acetate at a