

Full Length Research Paper

Establishment and application of antioxidant capacity screening model of natural Chinese herbal medicines

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Ethylene was measured by gas chromatography to determine total oxyradical scavenging capacity of water extracts for 20 kinds of natural Chinese herbal medicines. The result showed that the water extracts of 20 kinds of Chinese herbal medicines had good antioxidant capacity. Among them, Green tea, *Laminaria japonica* Aresch, *L. japonica* Thumb, *Carthamus tinctorius* L., *Semen Persicae*, *Myristica fragrans*, *Scutellaria baicalensis* Georgi had higher TOSC value and stronger antioxidant capacity. TOSC method was a rapid and sensitive method with better repeatability and it was applicable to preliminary screening of antioxidant capacity of Chinese herbal medicines.

Keywords: Chinese herbal medicines, antioxidant capacity, gas chromatography, total oxyradical scavenging capacity.

INTRODUCTION

With the deepening of theory and research of oxygen free radicals and antioxidants, applications of natural antioxidants are concerned more and more. Especially as some synthetic antioxidants have some toxicity in use (Schildermann et al., 1995). People more depend on natural antioxidants. Therefore, return to nature has been a big trend at present. Some studies show that traditional Chinese herbal medicines and natural plants contained a lot of free radical scavengers such as flavonoids, terpenoids, phenol, alkaloids, VitE, VitC etc. and they have strong radical scavenging and anti-aging effects (Beckman; Brett et al., 2010; Le Ying et al., 2011). In china, there are various kinds of Chinese herbal medicines, which is a giant treasury of natural antioxidants. However, as ingredients of Chinese herbal medicines are very complex, there are many difficulties for development of Chinese herbal medicines.

Traditional Chinese medicine uses some theories such as "4 Xing and 5 Tastes", "Ascending and Descending, Floating and Sinking", "Channel T", "18 Fan and 19 Wei" to evaluate effects of Chinese herbal medicines, namely

"Activating blood circulation to dissipate blood stasis", "Nourishing Yin and Supplementing Blood", "Invigorating Kidney and Strengthening Yang", "Prolonging life" and "Clearing Heat and Detoxicating" so on. This is the summary previous experiences and wisdoms, and it is nonrepresentational, without a quantitative index. At present, there are many detection methods of antioxidant capacity of natural antioxidants, including TOSC method (Total Oxyradical Scavenging Capacity) (Winton et al.,1998), FRAP method (Ferric ion Reducing Antioxidant Power) (Benzie and Strain, 1996), TEAC method (Trolox Equivalent Antioxidant Capacity) (Miller et al.,1993), DPPH method (2,2'-Diphenyl-1-picrylhydrazyl Radical Scavenging Capacity) (Brand-Williams W et al.,1995), ORAC method (Oxygen Radical Absorbance Capacity) (Cao et al.,1993), TRAP assay (Total Peroxyl Radical-trapping Antioxidant Parameter Assay) (Ghiselli et al.,1995; Wayner et al.,1985), electron spin resonance spin-trapping technique, Chemical luminescence, photochemical reduction method of nitroblue tetrazolium (NBT) etc, while electron spin resonance spin-trapping technique, Chemical luminescence method, nitroblue tetrazolium(NBT) photochemical reduction method are the methods only taking O₂^{·-} or ·OH simple radical as the substrate, and their shortcomings lie in that it is very difficult to comprehensively reflect actual antioxidant

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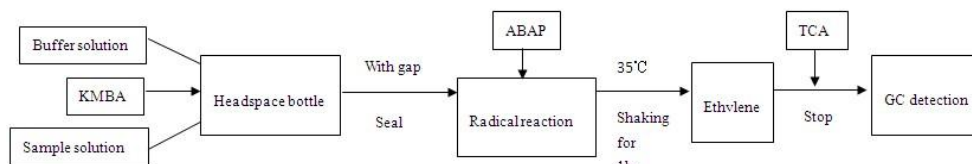


Figure 1. Flow diagram of activated oxygen Generation and ethylene preparation and detection.

capacity of natural antioxidants. This paper selected 20 kinds of Chinese herbal medicines and referred to the headspace gas chromatography (HS-GC) method established by Winston et al for determining TOSC (Winton et al., 1998) to evaluate hazardness of environmental pollutants to aquatic organisms and used it as a evaluation measure for antioxidant capacity of Chinese herbal medicines.

MATERIALS AND METHODS

Instruments and equipments

Gas chromatograph (Shimadzu GC-14B, with FID); C-R6A data processor (Shimadzu); Constant-temperature oscillating water bath (DKZ-2 type of Shanghai Keqi company, accuracy of $0.1\pm^{\circ}\text{C}$); Plant grinder (Wenling Grain Testing Factory FSF-90), Tissue homogenate machine (Shanghai specimen model factory DS-1); Constant-temperature water bath (Jiangsu Dongtai Electrical Appliance Factory, HHS type); Reflux extraction device (self-made); Vacuum suction device; Glass syringe, 1 ml Headspace device (a hard glass bottle (10ml); an isolation gasket with a plastic screw cap and the inner lining of Teflon ethylene film; one puncture hole of ϕ 3 mm at the center of bottle cap. Before use, the glass bottle was washed and dried at 100°C for 30 to 60 min, and the isolation gasket was boiled with distilled water for 30 min, washed with double distilled water and dried for use).

Chemical reagents

2,2' - azo bis (2 - amidino propane dichloride) (ABAP) (Wako chemical Co.), 4 - methylthio - 2 - Ketobutyric sodium (KMBA) (Sigma), 0.01M sodium phosphate buffer solution (PBS, PH was adjusted to 7.4 with phosphoric acid), trichloroacetic acid (TCA).

Experimental raw materials

(Various Chinese herbal medicines were purchased from Herbal Pieces Factory of Zhejiang Chinese Medicine University). Chrysanthemum morifolium Ramat; Indian Buead; Kelp; Semen Nelumbinis; Barbary Wolfberry Fruit; Lotus Plumule; Green Tea; Nutmeg; Baical Skullcap Root; Safflower; Liquoric Root; Chinese Angelica; Parslane Herb; Honeysuckle Flower; Hawthorn Fruit; Ginger P.E; Ephedra Herb; Giant Knotweed Rhizome; Chinese Date; Peach Seed.

Establishment of screening model

TOSC detection principle

In a closed system (headspace device), pyrogenation of ABAP produced a large amount of oxygen free radicals centering around

carbon. KMBA could be oxidized into ethylene under action of oxygen free radicals, and GC method was used to detect ethylene content. In the presence of antioxidants, antioxidants could compete with KMBA in combination with oxygen free radicals to decrease production of generated ethylene. Therefore, TOSC value of tested sample could be calculated from the reduction amount of generated ethylene. Calculation formula of TOSC could be showed as following:

$$TOSC = 100 - \frac{\int SA}{\int CA} \times 100$$

Where, $\int SA$ and $\int CA$ respectively represented the amounts of generated ethylene in the sample tube and the control tube (expressed as integral value of chromatographic peak area).

In the system, possible free radical reactions included:

1. $R_2N_2H \rightarrow \cdot OOH(R)$ or $\cdot OH(R)$
 2. $CH_3S-(CH_2)_2-CO-COOH + \cdot OOH(R)$ or $\cdot OH(R) \rightarrow (CH_3)_2OS + CH_2=CH_2 \uparrow + CO_2 + (R)HOO\cdot$ or $(R)HO\cdot$
- Where: R_2N_2H' , $\cdot OOH(R)$ and $\cdot OH(R)$ respectively represented ABAP, lipid peroxy radical and lipid hydroxy radical.

Generation of activated oxygen and preparation and detection system of ethylene

0.1 mmol/L PBS buffer solutions (pH 7.4) was used to prepare 0.2 mol/LABAP and 0.25 mmol/L KMBA (the two solutions were temporarily prepared before use and stored in the dark place at 4°C). Corresponding agents were added into the headspace bottle ((KMBA 0.8 ml + PBS 0.1 ml) for control tube, (KMBA 0.8 ml + sample solution 0.1 ml) for sample tube). After the headspace bottle was sealed with a cap, 0.1 ml ABAP was injected with a syringe until the total volume of the solution reached 1ml. The solution was shaken for 60min in water bath at 35°C . Subsequently, 0.3 ml TCA of 30% was immediately injected for stopping reaction, and 0.5 ml headspace gas was extracted with glass syringe of 1ml for gas chromatographic analysis (Winton et al., 1998; Cohen et al., 1987) Figure 1.

Chromatographic conditions

GDX-502 or 10% SE-30 packed column (2 m \times 3 mm, glass column); temperatures of sample injector, column and detector were respectively 150, 60 and 200°C . The detector was flame ionization detector (FID), and flow rates of carrier gas (N_2), H_2 and air were respectively 40, 50 and 500 ml/min.

Preparation of ethylene standard curve

Different volumes of 100 ng/ml (20°C , 1 atm) ethylene standard gases (0, 0.56, 1.4, 2.8 and 4.2 ml respectively represented 0, 2, 5, 10 and 15 nmol of ethylene amount in 10 ml system) were respectively injected with 1 to 5 ml airtight plastic syringes into 5 sealed

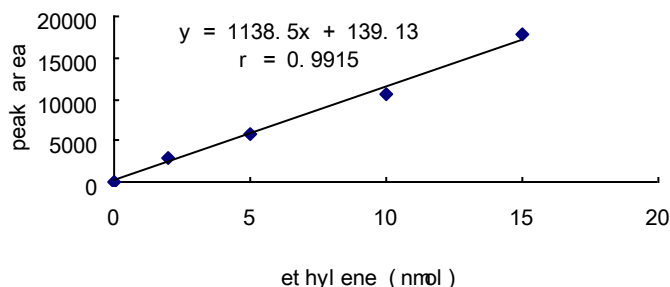


Figure 2. Standard curve of ethylene detection.

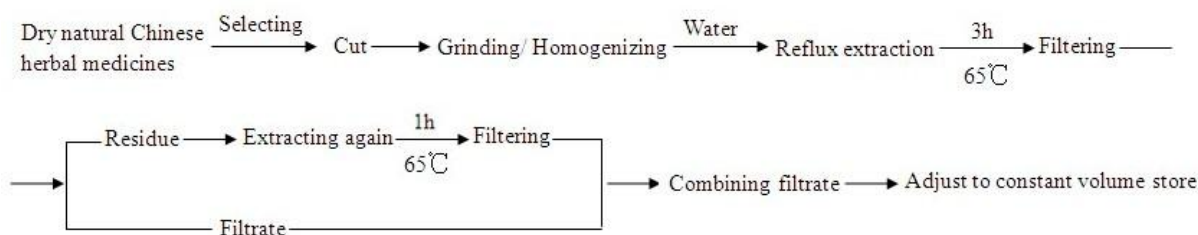


Figure 3. Sample detection.

headspace bottles with caps (each bottle contained 1 mlPBS). According to Figure 1, 0.5 ml headspace gas was extracted for gas chromatographic analysis. Curve of ethylene amounts versus integral values of chromatographic peak area was plotted, and a regression equation was obtained

$$Y=1138.5X+139.13, r=0.9915(n=5).$$

Linear range was 0.01 to 0.75 nmol, and the minimum detectable limit of ethylene was 0.01 nmol. (Figure 2)

Application of screening

Sample pretreatment

Dry natural Chinese herbal medicines were selected, cut and ground (those couldn't be ground were crushed by tissue homogenate machine). 50 g Chinese herbal medicine was weighed and extracted by reflux with distilled water for 3 h in 65°C water bath. After filtration, the residue was extracted for 1 h in the same conditions (totally, 200 ml distilled water was added). Filter paper was used for filtration. The two times of filtrate were combined, and the solution volume was adjusted to 250 ml (1 ml sample solution equaled to 0.2 g raw Chinese herbal medicine). At last, the solution was stored away from light in the refrigerator for use. In case of detection, the solution was diluted. The process was shown in Figure 3.

Sample detection

Water extract of sample prepared in Sample pretreatment was taken and diluted to a certain dilution ratio with distilled water (in this experiment, the dilution ratio of 5 was used, and the concentration was 0.01 mg/ml). According to Figure 1, sample

injection, reaction and detection were carried out.

RESULTS AND DISCUSSION

Selection of chromatographic conditions

Ethylene is a small polar molecule substance, and its boiling point is -40°C. At normal temperature, it is a gas. The experiment compared GDX-502 packed column with 10% SE-30 packed column. Many trials proved that good separation efficiency could be obtained in the chromatographic conditions (Figure 4 and 5). For SE-30 column, time ethylene peak was at 0.32 min, and there was still an unknown peak at 1.8 min, which delayed analysis time. In absence of GDX-502 column, SE-30 column could be used alternately (Ruben t'Kindta et al., 2009).

Recovery rate test

In 9 blank detection systems (PBS buffer solution), high, medium and low doses of ethylene (2, 5, 10nmol/10ml ethylene standard) were respectively injected. Detections were carried out according to Figure 1. After processing, the average recovery rate was 97.2%.

Repeatability test

In 10 same blank detection systems, ethylene standard of 2nmol/10ml was respectively injected. According to

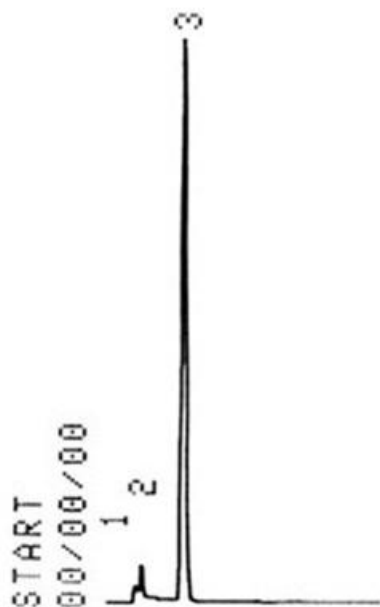


Figure 4. Chromatogram of TOSC detection of the control tube 1,2-unknown peaks; 3-ethylene (GDX)

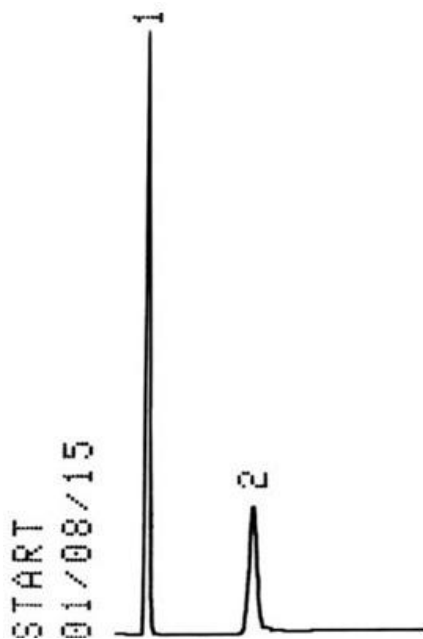


Figure 5. Chromatogram of TOSC detection of the control tube 1- Ethylene peak; 2-unknown peak (SE-30)

Figure 1, operations were carried out. As a result, integral mean value of chromatographic peak area was 1637.8 ± 160.37 , and relative standard deviation was 4.8% (n=10), complying with requirements of headspace-gas chromatography.

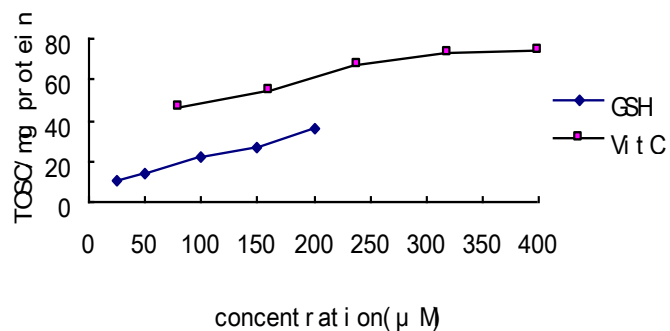


Figure 6. Dose effect relationships of GSH and VitC with TOSC.

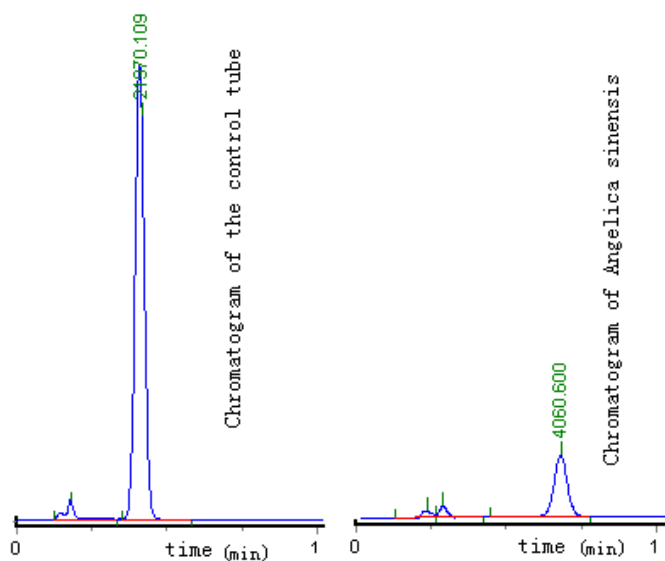


Figure 7. Chromatograms of ethylene selection of the control tube and water extract of Angelica sinensis.

Adaptability test of detection system

For testing response situations of detection system to the antioxidant capacity of Chinese herbal medicines, TOSC values of different antioxidants GSH and Vit C added into the reaction system were detected (Jan Thomas Michaelson et al., 2009). The result showed that within applied concentration range, the antioxidant had dose effect relationships with TOSC. As shown in Figure 6, when GSH was less than 320 μM, the two had a linear relationship, and correlation coefficient was 0.9973. When concentration of Vit C was 80 μM, Vit C and TOSC had a better linear relationship, and correlation coefficient was 0.9854. TOSC rose with increase of antioxidant concentration, and GSH and Vit C had linear relationship with TOSC in a certain concentration range, indicating TOSC could reflect the antioxidant capacity of antioxidants.

Table 1. TOSC values of 20 kinds of Chinese herbal medicines.

Latin Name	English name	TOSC
<i>Flos Chrysanthemi</i>	Chrysanthemum morifolium Ramat	95.7929
<i>Poria cocos</i> (Schw.) Wolf	Indian Buead	55.2386
<i>Laminaria japonica</i> Aresch	Kelp	99.6022
<i>Lotus Seed</i>	Semen Nelumbinis	86.5770
<i>Fructus Lycii</i>	Barbary Wolfberry Fruit	94.6899
<i>Plumula Nelumbinis</i>	Lotus Plumule	94.6373
<i>Camellia sinensis</i>	Green Tea	100.0000
<i>Semen Myristicae</i>	Nutmeg	97.9673
<i>Radix Scutellariae</i>	Baical Skullcap Root	97.4346
<i>Flos Carthami.</i>	Safflower	98.7436
<i>Radix Glycyrrhizae</i>	Liquoric Root	89.1166
<i>Radix Angelicae Sinensis</i>	Chinese Angelica	67.2449
<i>Herba Portulacae</i>	Parslane Herb	62.8551
<i>Flos Lonicerae</i>	Honeysuckle Flower	98.9392
<i>Fructus Crataegi</i>	Hawthorn Fruit	92.4252
Rhizoma Zingiberis Recens	Ginger P.E	89.0299
<i>Herba Ephedrae</i>	Ephedra Herb	95.5149
<i>Rhizoma Polygoni Cuspidati</i>	Giant Knotweed Rhizome	93.0307
<i>Fructus Jujubae</i>	Chinese Date	95.1118
<i>Semen Persicae</i>	Peach Seed	98.4168

Selection of raw material extraction method

There are many methods of extracting antioxidant active ingredients of Chinese herbal medicines, and the common method is solvent extraction method. The solvents include Water, ethanol, methanol, chloroform, ether, acetic acid, acetone, benzene, petroleum ether etc. Among them, 95% ethanol is the most frequently used. The experiment applied equal amount of water and 95% ethanol and compared their extraction situations. The result of equivalent dose of any sample solution showed that the later had no obvious ethylene peak. Considering that ethylene was associated with the reducibility of alcohol (i.e., alcohol could capture free radicals). This screening model wasn't possibly extracted with alcohol. So, this study used water for extraction.

Detection results of water extract of Chinese herbal medicines samples

Ethylene peaks of detected control tube and Angelic sample tube were shown in Figure 7. It could be obviously found from Figure 7 that after water extract of sample was added, peak area and peak height ethylene greatly reduced, suggesting that the amount of ethylene generated by reaction system greatly reduced. It was shown that water extract of the sample had better antioxidant effect. At the same time, it was also indicated that this detection method had higher sensitivity. TOSC

values of 20 kinds of water extracts of Chinese herbal medicines were shown in Table 1.

Data in Table 1 showed that total oxyradical scavenging capacity (TOSC) of various Chinese herbal medicines were strong. Among them, TOSC value Green tea reached 100, and its antioxidant capacity was the strongest. Possible reason lay in that it contained tea polyphenol with the stronger antioxidant activity. *Laminaria japonica* Aresch was in the second place, and its TOSC value was 99.6022, which was in line with the literature (Shiwang and She, 1995). Next, TOSC values of *Lonicera japonica* Thumb, *Carthamus tinctorius* L., *Semen Persicae*, *Myristica fragrans* and *Scutellaria baicalensis* Georgi all were more than 97%, and they had the higher antioxidant activity. This result was not in line with the result obtained by Hu Bolu et al by use of DPPH method (Hu Bolu et al., 2000). Their result showed that free radical scavenging capacity of *Scutellaria baicalensis* Georgi and *Ephedra sinica* Stapf was stronger than that of tea polyphenol. In this study, *Scutellaria baicalensis* Georgi and *Ephedra sinica* Stapf were respectively in the 7th and 9th places. The difference reason possibly lay in that production place, processing condition, storage time and experimental method of purchased Chinese herbal medicines were different. As active ingredients of Chinese herbal medicine antioxidation were complex, and different methods of screening antioxidant capacity of Chinese herbal medicines used different bases to explain the results, different results were possibly obtained. For reliability of detection result of TOSC method, it required

further research and exploration, such as expanding screening range, using comparison with the positive reference and directly determining antioxidant ingredients by combining chromatography and spectroscopy so on.

Conclusions

This study establishes TOSC detection method. The method has rapid, sensitive and high-resolution advantages similar to GC method (Li et al., 2011), and it has good recovery rate and good repeatability. It considers effects of all substances reacting with oxygen radical, overcomes the problem of determination deficiency of single antioxidant component and reflects total antioxidant capacity of the system by means of conversion of ethylene preparation system. In the detection system, KMBA can also accept ethylene caused by attack of other free radicals (for example, hydroxy radical generated by Fenton reaction, Peroxynitrite radical generated by pyrogenation of 3-morpholinosydnomine) (Lionel et al., 2004), which can expand the application range of TOSC method. Therefore, it is feasible to carry out preliminary screening of antioxidant ingredients of a great number of relevant Chinese herbal medicines in a shorter time, which will help to increase the strength and depth of natural antioxidants product development and contribute to promote the modernization and internationalization of traditional Chinese medicine.

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