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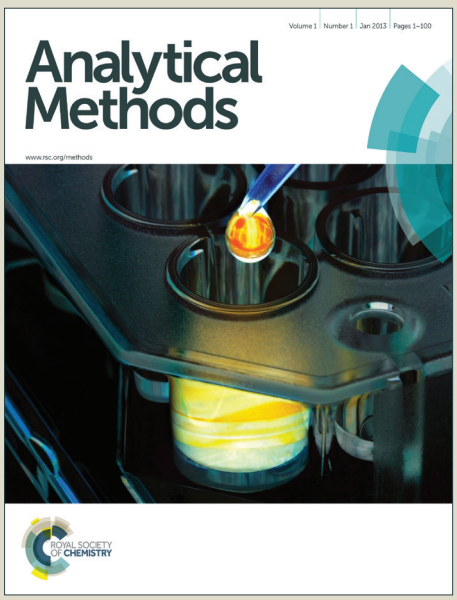

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Rapid assay for testing superoxide anion radical scavenging activities to natural pigments by ultra-high performance liquid chromatography-diode-array detection method

Lu Wang[a,b], Shu Liu[a], Zhong Zheng[a], Zifeng Pi[a],Fengrui Song[a*], Zhiqiang Liu[a]

[a] National Center for Mass Spectrometry in Changchun, Jilin Province Key Laboratory of Chinese Medicine Chemistry and Mass Spectrometry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun 130022, China

[b] University of Chinese Academy of Sciences, Beijing, 100049, China

*Corresponding author: F. R. Song, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun 130022, China.

Tel.: (086) 0431-85262044

Fax: (086) 0431-85262044

E-mail address: songfr@ciac.ac.cn

ABSTRACT

As a result of the donation of one electron, superoxide anion radicals ($O_2^{\bullet-}$) are produced in vivo which are closely linked with several human diseases. In the past decades, some analytical methods for the determination of $O_2^{\bullet-}$ scavenging capacity have been established. The most common methods in vitro are spectrum-based microplate screening assay using nitrobluetetrazolium (NBT) or cytochrome c as target/probe for evaluating $O_2^{\bullet-}$ scavenging activity. The target/probe is spectrophotometrically monitored at Ultraviolet-Vis (UV-Vis) region in the analysis of the samples with restraining UV-Vis absorption, Nevertheless, the result of these methods were severely compromised when they were applied to analyze the samples with strong absorption in the visible region, such as natural pigments. To solve the problems, a simple and rapid assay combined with a new probe, a highly water-soluble tetrazolium salt, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl) -2H-tetrazolium sodium salt (WST-1) and ultra-high performance liquid chromatography-diode-array detection（UPLC-DAD）method was developed. Above all, this method could be adapted to samples of strong absorption in the visible region. In the study, the superoxide anion radical scavenging activities of various natural pigments were evaluated.

KEYWORDS WST-1, Superoxide anion radicals, UPLC-DAD, natural pigments, Scavenging ratios

INTRODUCTION

Over the past decades, the formation of reactive oxygen species (ROS) has been implicated in the pathogenesis of several human diseases such as diabetes mellitus, chronic inflammation, neurodegenerative disorders and certain types of cancer[1-3]. In this context, the researches of reactive oxygen species (ROS), including superoxide anion radicals ($O_2^{\bullet-}$), hydroxyl radicals ($HO^{\bullet}$) and peroxyl radical ($ROO^{\bullet}$), have received increasing attention lately. The superoxide anion radicals ($O_2^{\bullet-}$), generated by the donation of one electron to oxygen, are one of the most important and biologically relevant ROS radicals in living organisms. And the superoxide anion radicals arise either from several metabolic processes or following oxygen activation by irradiation[4]. As a kind of most active reactive oxygen species (ROS), superoxide anions radicals ($O_2^{\bullet-}$) are involved in a lot of physiological and pathological processes[5]. Thus, a simple, rapid, and reliable in vitro analytical method is needed for the fast determination of antioxidant capacities of pure compounds or complex samples, such as food and natural products.

For measuring superoxide anion radical scavenging activities, a suitable system should be selected for generating $O_2^{\bullet-}$. Generally, there are two systems to produce superoxide anion radicals: xanthine/xanthine oxidase system and phenazine methosulphate (PMS) system in the presence of nicotinamide adenine dinucleotide (NADH). The formation reaction of superoxide anion radicals ($O_2^{\bullet-}$) is based on the catalysis of xanthine oxidase as follows[6]:

$$\text{Xanthine} + 2O_2 + H_2O \rightarrow \text{Uric acid} + 2O_2^{\bullet-} + 2H^+ \quad (1)$$

But both systems may produce error results if the related enzymes are inhibited

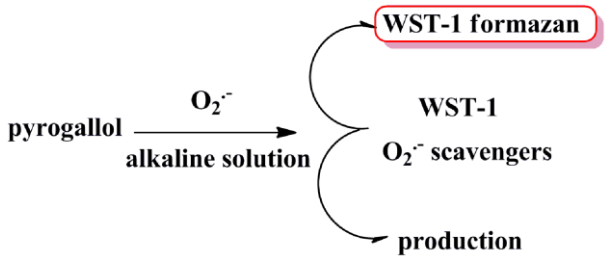
or interfered with $O_2^{\bullet-}$ generation. For example, many plant extracts could inhibit the activity of xanthine oxidase, so that the activity of xanthine oxidase is interfered with $O_2^{\bullet-}$ generation, which will cause false positive results. Liu et al. had reported that several herbal extracts could be used as $O_2^{\bullet-}$ scavengers, but these extracts might inhibit the activity of xanthine oxidase[7]. So, considering above the disadvantages for generating $O_2^{\bullet-}$ in enzyme system, 1,2,3-trihydroxybenzene (pyrogallol) was selected to generate $O_2^{\bullet-}$ in our study because pyrogallol can be autoxidized without enzyme under alkaline conditions[8]. Autoxidation system of pyrogallol efficiently avoid the interferences in the generation of $O_2^{\bullet-}$ like herbal extracts.

For testing superoxide anion radical scavenging activities of samples, in a method, there should also be a corresponding target analyte called probe/target for measuring the $O_2^{\bullet-}$ scavenging capacities of samples. The probe/target itself or its production for this purpose should be easily detected. For example, $O_2^{\bullet-}$ may reduce nitrobluetetrazolium (NBT) into formazan, which could be determined with spectrophotometric method at 560nm[9-10]. Also, NBT formazan could be measured by electron spin resonance (ESR) method[12]. As a probe, NBT system is sensitive, but unfortunately, NBT formazan is water-insoluble, *i.e.* in a suspended state, leading to the un-reliable results. At present, another widely used probe for the detection of $O_2^{\bullet-}$ is cytochrome c, but, the react ratio between cytochrome c and $O_2^{\bullet-}$ is too fast to control[11], thus this compound as a probe for $O_2^{\bullet-}$ is not ideal. In addition, although luminol or lucigenin are frequently applied as target/probe compounds, the quantitative analysis is performed by using chemiluminescence's (CL) method[13],

which is not suitable to us.

In this study, a new probe,2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium sodium salt (WST-1) was used for reacting with superoxide anion radicals to form a stable water-soluble compound (WST-1 formazan) which can be conveniently detected at 450nm by spectrophotometry. The reaction is as follows:

$$\text{WST-1} + 2O_2^{\bullet -} + H^+ \rightarrow \text{WST-1 formazan} + 2O_2 \qquad (2)$$



Scheme1. The principle of the method. Superoxide anion radical scavenging activity was accomplished by quantifying the probe (WST-1 formazan).

The generated WST-1 formazan is highly water-soluble (water-solubility is greater than 100mM). Xu et al. reported that WST-1 could be utilized as a probe for measuring the superoxide anion radical scavenging activities of samples by UV-Vis spectrometry method (microplate measurement) at 450nm[14]. However, many herb extracts and natural pigments have strong absorptions in the visible region (see Table 1), such as at 450nm, which could interfere with the above measurement. So the above method cannot be applied to measure the superoxide anion radical scavenging activities of these samples. As yet, the antioxidant activities of some natural pigments have been reported, such as flavonoids and carotenoids[15-16], but there is no analysis method for measuring the superoxide anion radicals scavenging activities of natural

pigments except for few studies to compare the antioxidant activities of a different type of natural pigments[26]. For these reasons, we developed the UPLC-DAD method to detect the superoxide anion radical scavenging activities of natural pigments. This method separated WST-1 formazan as the detection probe from other interferences in complex reaction system and overcame the shortcomings of spectrometry method. The specificity and accuracy of the measurement result were improved by chromatographic separation. Hence, the UPLC-DAD method could be adapted to these complex systems. This simple and rapid method could be used not only for natural pigments but also or herb extracts and other samples.

Table 1 Typical compounds of natural pigments that have strong absorptions in the UV-Vis region

	Typical compound	UV/Vis (λ_{max})	Source
Flavonoids	Anthocyanins	530nm	*Vaccinium*
	Safflower yellow A	403nm	*Carthamus tinctorius L.*
Polyphenols	Theaflavins	363nm	*Camellia sinensis*
	Curcuminoid	425nm	*Curcuma longa,C.zedoaria,C.huangsiyujin*
Quinones	Shikonin	516nm	*Lithosperrnum erythrorhizon*
Carotenoids	β-Carotene	455nm	*Ipomoea batatas* ,carrots and pumpkins
	Lutein	446nm	*Brassica oleracea* , *Spinacia oleracea*
	Crocin	440nm	*Crocus*, *Gardenia jasminoides*
	Astaxanthin	472nm	Microalgae, yeast, salmon, trout, krill, shrimp, crayfish and crustaceans
	Lycopene	472nm	*Solanum lycopersicum*
Alkaloids	Betanin	538nm	*Beta vulgaris*

EXPERIMENTAL

Standards and reagents

L-ascorbic acid as positive control and ammonium hydrogen carbonate (NH_4HCO_3) were purchased from Fluka (Buchs, Switzerland); natural pigments such as hydroxysafflor yellow A, curcumin, morin hydrate, lutein, shikonin, crocin Ⅰ, crocin Ⅱ ,β-carotene and gardenia yellow were taken from Shanghai Yuanye Bio-Technology Co., Ltd (Shanghai, China), 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium sodium salt (WST-1) as probe for measuring the $O_2^{\bullet-}$ scavenging capacities of samples was obtained from Nanjing Robiot *Co*., *Ltd.* (Nanjing, China); 1,2,3-trihydroxybenzene (pyrogallol) for generating $O_2^{\bullet-}$ was supplied by Aladdin(Shanghai, China). Acetonitrile of HPLC grade was supported by Fisher Scientific (Loughborough, UK). Deionized water was prepared using the Milli-Q water purification system (Milford, MA, USA). All of the other reagents were analytical grade.

Methods

Preparation of samples and their absorption spectra analysis

2mM stock solutions of natural pigment compounds were prepared by dimethylsulfoxide (DMSO). In order to maintain pH 9.3 of the reaction system, the stock solutions were diluted to prepare different concentration solutions by 50mM NH_4HCO_3-$(NH_4)_2CO_3$ buffer solution, respectively. All the sample solutions were filtered through a 0.22μM membrane. Then, the absorption spectra of these sample solutions were measured by GENESYS 10S UV-VIS spectrophotometer.

The superoxide anion radical ($O_2^{\bullet-}$) reaction system with the WST-1

The reaction system was based on that reported by Xu et al. [14] The 50mM

NH_4HCO_3-$(NH_4)_2CO_3$ buffer solution at pH 9.3was prepared in deionized water, and its pH value was regulated with ammonium hydroxide. The pyrogallol was dissolved in deionized water with 1mM HCL for preventing oxidation. 0.5mM ethylene diaminetetraacetic acid (EDTA) and 50μM WST-1 were prepared in the buffer solution. 40μL WST-1 was added to the sample (or corresponding solution) in a final volume of 160μL in 50mM ammonium hydrogen carbonate buffer, 0.5mM EDTA, pH 9.3. Reaction was started by adding 40μL 1mM pyrogallol. The experiment was proceeded on the 96-well microplate under 37°C, and was terminated by acetic acid at 12 min.

Spectrophotometric method for reaction progress monitoring

The reaction progress of the reaction system could be monitored by measuring the absorption values of WST-1 formazan at 450nm with TECAN GENiosMicroplate Reader. Since *L*-ascorbic acid has a quite weak absorption near 450nm, it was selected as positive control to monitor the reaction progress. The same concentration solution of *L*-ascorbic acid was prepared by pure NH_4HCO_3-$(NH4)_2CO_3$ buffer solution and the buffer solution containing 10% DMSO, respectively.

UPLC–DAD method development

The UPLC–DAD experiments were performed on a Waters ACQUITY^{TM} UPLC system equipped with a diode-array detector (DAD) and an autosampler (Waters Corp., Milford, MA, USA). A Waters ACQUITY^{TM} UPLC BEH C18 Column (1.7μm, 2.1 mm×50 mm) (Milford, MA, USA) was used for separation at 30 ℃. The mobile phase consisted of water (A) and acetonitrile (B), and the flow rate was set at 0.3 mL

min^{-1}. The gradient elution was employed for separation of WST-1 formazan from other interferences. Gradient elution was as follows: initially, 50% to 70% B in 1 min, 70% to 100% B at 1 to 2 min, 100% B at 2 to 6 min. The injection volume was 10μL. UV–Vis spectra were recorded in the range of 210–500 nm, and the chromatograms were acquired at 450nm (WST-1 formazan). The superoxide anion radical scavenging ratio was calculated according to the following formula:

$$\text{Scavenging ratio (\%)} = \frac{(A_0 - A_1)}{A_0} \times 100 \qquad (3)$$

Where A_0 is the peak area of the WST-1 formazan of the blank control, and A_1 is the peak area of the WST-1 formazan in presence of the samples, *i.e.*, standards.

Precision

Precisions in intra-day and inter-days were evaluated by determining the $O_2^{\bullet-}$ scavenging ratios of *L*-ascorbic acid as positive control at three different concentrations (six replicates for each concentration), and expressed as the relative standard deviation (RSD). The RSD of HPLC method should not exceed 3%.

RESULTS AND DISCUSSION

UV-Vis spectra of the samples

As natural pigments, all the sample solutions in this research had deep colors and have strong absorption in visible region (400-800nm). Fig.1A shows the UV-Vis spectra of crocin Ⅰ,crocin Ⅱ and gardenia yellow. The similar profiles reveal the common backbone of the structures. Actually, crocin Ⅰ and crocin Ⅱ are the main compositions of crocus pigment in the gardenia yellow which have a strong

absorption at 440nm. The UV-Vis spectrum of shikonin is shown in Fig.1B. As a kind of the anthraquinones, shikonin is an important pharmaceutical ingredient and has the activities of hemostatic and anti-inflammatory. Shikonin has benzene ring and diketone structure which probably result in an absorption spectrum at 520nm. Morin hydrate is a kind of widely distributed plant pigment and it belongs to flavonoids. Lutein and β-carotene, two of the carotenoids, have closely related to immune defenses and reproductive health[16]. As shown in Fig.1E (lutein) and Fig1G (β-carotene), their maximum absorption spectra are near 450nm. Curcuminoids are safe natural yellow pigments used as food coloring agents and traditional drugs with a variety of biological functions such as antitumor, anti-inflammatory and antioxidant activities[17-18]. Fig.1D reveals that the characteristic absorption of curcumin is 430nm. Hydroxysafflor yellow A (Safflomin A) has the structure of chalcone with glycoside, and its maximum absorption is at 400nm. Above all, almost all the pigment samples have the maximum absorption spectra near 450nm, which overlapped with the maximum absorption at 450nm of WST-1 formazan as the probe. These absorption will seriously interfere with the analytical results of $O_2^{\bullet -}$ scavenging activities of these samples by UV-Vis method, and lead to the un-reliable results. Thus, ultraviolet-visible spectrophotometry method could not be applied to the samples which have strong absorption in the 400-500nm region like natural pigments. However, the UPLC-DAD method could overcome the above problems, and it is simple and rapid for testing superoxide anion radical scavenging activities of compounds with strong absorption in the visible region.

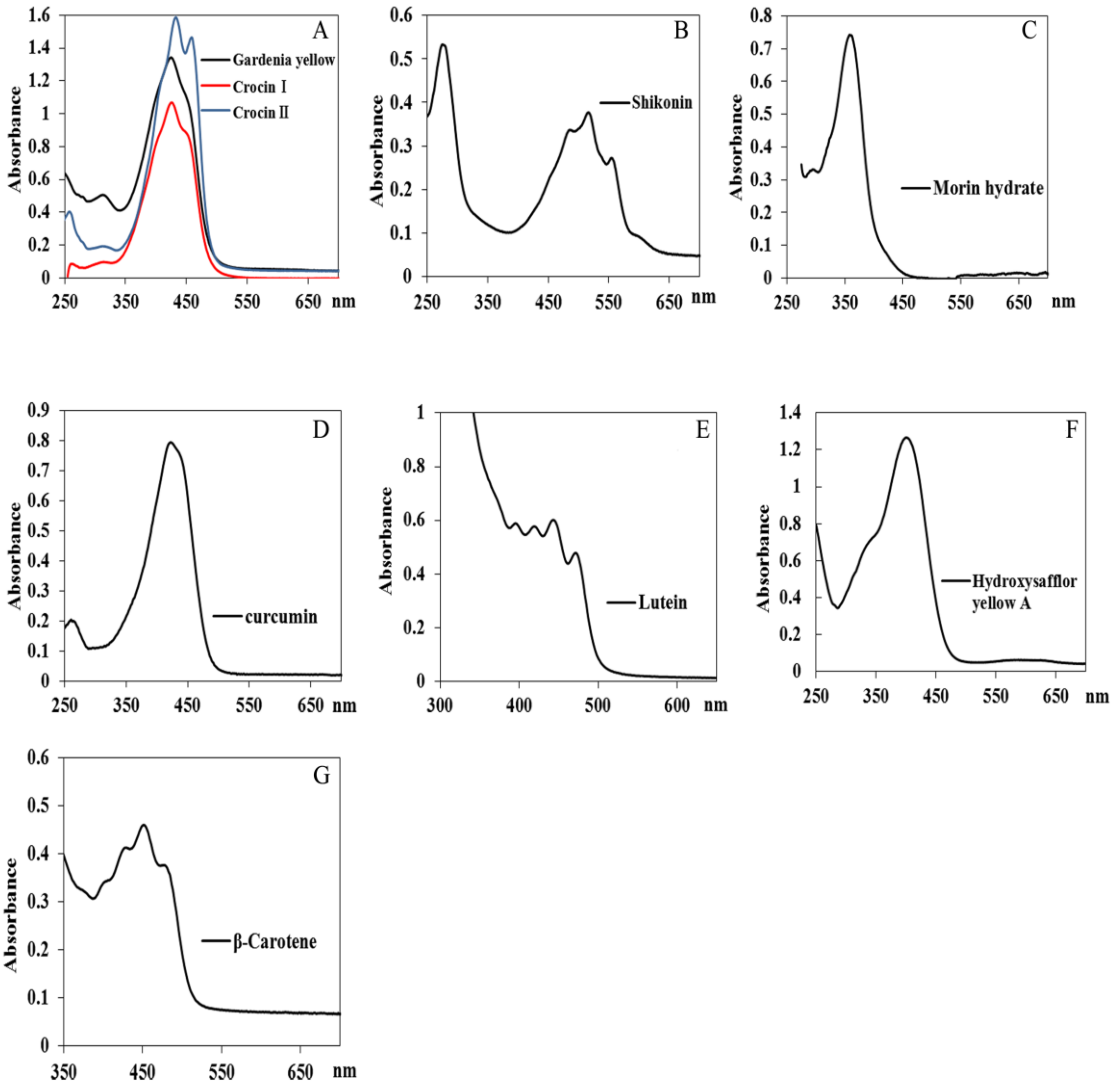


Fig.1 Characteristic UV-Vis spectra of different samples.

Determination of the optimal reaction time in different reaction systems

Previous studies have indicated that pH of the reaction system plays a crucial role in the reaction system. The change of pH should influence the results of the superoxide anion radical scavenging activities[14]. *L*-ascorbic acid was chosen as a positive control sample to measure absorbance of the system, namely, the content change of WST-1 formazan. It is worth noting that some samples are easily dissolved in the NH_4HCO_3-$(NH_4)_2CO_3$ buffer solution, nevertheless, some other samples being low

polar could be dissolved in DMSO rather than in the buffer solution. So Fig.2 shows the effect of DMSO on the absorbance-time curve of *L*-ascorbic acid in different reaction systems. Fig.2A indicated that the slight different absorbance values of *L*-ascorbic acid between two reaction systems, *i.e.*, pure buffer solution system and the buffer solution with 10% DMSO system. In the same reaction time, the absorbance values of *L*-ascorbic acid in buffer solution containing 10% DMSO were lower than that the values of *L*-ascorbic acid in the pure buffer solution. At the same time, the absorbance-time curves of *L*-ascorbic acid showed that the production rates of WST-1 formazan within 1-10 minutes were lower. The production rates reached a maximum from 10-20 minutes, which might relate to the formation rates of superoxide anion radical ($O_2^{\bullet-}$)[19]. The $O_2^{\bullet-}$ scavenging ratios of *L*-ascorbic acid can be observed from Fig.2B, in which, the maximum value of the scavenging ratios of *L*-ascorbic acid appeared in 10 minutes to 20 minutes. What's more, the $O_2^{\bullet-}$ scavenging ratios of *L*-ascorbic acid in the buffer solution with 10% DMSO was lower than that of *L*-ascorbic acid in the pure buffer system. The reason might be the pH slight change of the reaction system as DMSO added to the buffer. The optimal reaction times of the reaction systems were 14min and 12 min in buffer（containing 10% DMSO)and in the pure buffer solution, respectively (see Fig.2B).

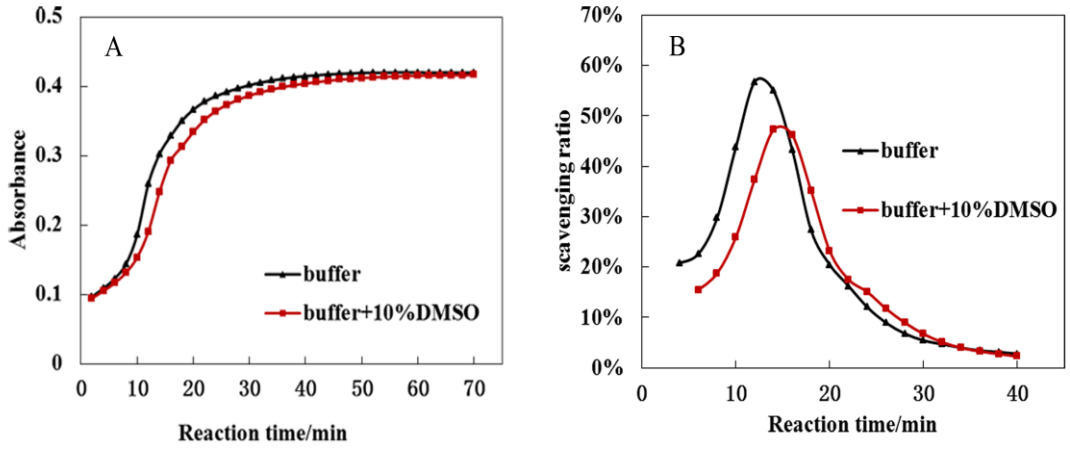


Fig.2 (A) The absorbance-time curves of *L*-ascorbic acid in different reaction systems; (B) The scavenging ratio–time curves of *L*-ascorbic acid in different reaction systems.

Validation of the proposed UPLC-DAD method

With the autoxidation of pyrogallol in alkaline solution, more and more $O_2^{\bullet-}$ generated in the reaction system. Since $O_2^{\bullet-}$ scavengers (antioxidants) reacted with $O_2^{\bullet-}$, WST-1 had to compete with $O_2^{\bullet-}$ scavengers (antioxidants) and the products (WST-1 formazan) decreased (see Eq.(1)). The principle of the method is shown in Scheme 1. Thus the change of WST-1 formazan represented the consumption of superoxide anion in the reaction mixture through the absorbance changes of the reaction system at 450nm[7]. The proposed UPLC-DAD method was used to evaluate $O_2^{\bullet-}$ scavenging ratios *of* *L*-ascorbic acid being as the superoxide anion radical scavenger. Fig.3 is the UPLC-DAD chromatogram of WST-1 formazan at 450nm. Fig.3B shows that the retention time for WST-1 formazan was 0.6-0.65min and the peak of WST-1 formazan was clearly separated from the others. Compared with Fig.3B, the peak area shown in Fig.3A decreased, because superoxide scavenger (*L*-ascorbic acid) was added into the reaction system to react with some superoxide

anion radicals. As an oxidant probe, WST-1 could be reduced to WST-1 formazan by reductant, *L*-ascorbic acid. Hence, A_0 is the peak area of WST-1 formazan in the blank control (see Fig.3B), and A_1 is the peak area of WST-1 formazan in the presence of samples (see Fig3A). (A_0–A_1) presents the decrease of superoxide anion (see Fig.3C) and (A_0-A_1)/A_0 presents the scavenging ratio (see Eq.(2)). So based on the change of the peak areas, the $O_2^{\bullet-}$ scavenging ratio of *L*-ascorbic acid at 0.25mM was 58.19%, which is consistent with those of previous studies[20]. Fig.4 shows the minor differences of $O_2^{\bullet-}$ scavenging ratio between the UPLC-DAD method and the UV-Vis spectrophotometry method, of which, the IC_{50} values of *L*-ascorbic acid were 0.17mM and 0.18mM, respectively. The validity and feasibility of UPLC-DAD method had been further verified by experimental results. Above all else, the method was simple and could be applied to measure superoxide anion radical scavenging activities of compounds which had strong absorptions in visible region.

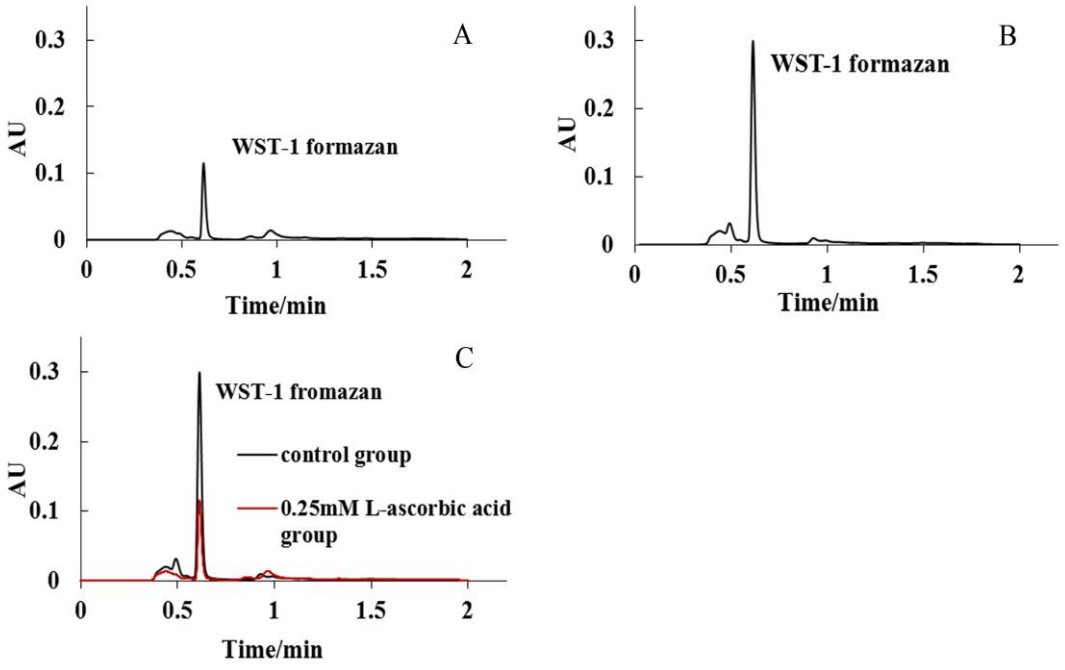


Fig.3 UPLC-DAD chromatograms of WST-1 formazan at 450nm: (A) reaction mixture in the presence of 0.25mM *L*-ascorbic acid ;(B) the control (without samples) ;(C) the overlay chromatograms of A and B.

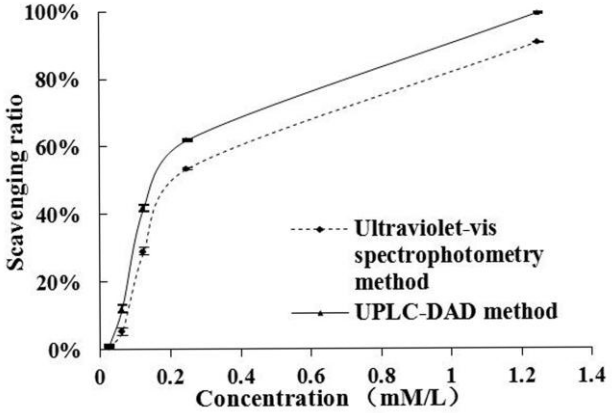


Fig.4 $O_2^{\bullet-}$ scavenging ratios comparisons of UPLC-DAD method and UV-Vis spectrophotometry method

Precision

The precisions of the UPLC-DAD method are shown in Table2. The intra-day and inter-day precisions of the method ranged from 0.22% to 2.87%, respectively. Obviously, the method has well reproducibility.

Table2 Precisions of the UPLC-DAD method (N=3)

Concentration of *L*-ascorbic acid (mM)	Scavenging ratio% Intra-day	RSD%	Scavenging ratio% Inter-day	RSD%
0.125	41.93	1.08	42.32	1.92
0.25	58.19	0.23	58.42	1.03
0.5	65.60	0.22	66.74	2.87

Applying UPLC-DAD method to evaluate the $O_2^{\bullet-}$ scavenging activities of natural pigments

$O_2^{\bullet-}$ scavenging activities of natural pigment were expressed as IC_{50} values (50% scavenging concentration). Higher IC_{50} value of a sample indicates its lower antioxidant effectiveness. More than five concentrations of each sample were tested for the $O_2^{\bullet-}$ scavenging ratios and the data were analyzed by Graphpad Prism 5. The data were recorded as mean ± standard deviation and the IC_{50} values of all natural pigments are shown in Table 3.

Table 3 IC_{50} values and scavenging ratios (%) of natural pigments for $O_2^{\bullet-}$ scavenging activities (mean ± SD, N = 3).

Natural pigments	IC_{50}（mM）	Scavenging ratio%（0.5mM）
L-ascorbic acid	0.219±0.004	73.76±1.20
Lutein	0.099±0.002	67.68±1.41
Curcumin	0.244±0.018	56.50±1.19
Morin hydrate	0.437±0.021	50.20±0.64
Hydroxysafflor yellow A	>0.5[a]	38.14±2.23
Crocin Ⅱ	>0.5[b]	38.34±0.21
Shikonin	>0.5[c]	34.78±0.08
Crocin Ⅰ	>0.5[d]	25.30±1.67
β-Carotene	>0.5[e]	24.56±2.11
Gardenia yellow (E500)	0.403±0.022mg/mL	-

[a-e] The IC_{50} values of natural pigments exceeded 0.5mM, and their scavenging ratio (%) at 0.5mM were listed in the right column of the table.

On the basis of the structures, the samples could be broadly classified as flavonoids (hydroxysafflor yellow A and morin hydrate), carotenoids (lutein,β-carotene, crocin Ⅰ and crocin Ⅱ), anthraquinones (shikonin) and diketones (curcumin). According to previous studies, the $O_2^{\bullet-}$ scavenging activities of would enhance with increasing the numbers of hydroxyl groups in ring B[21].In this study, the IC_{50} value of morin hydrate is 0.437mM. While, the IC_{50} value of hydroxysafflor yellow A is higher than 0.5mM, indicating that the $O_2^{\bullet-}$ scavenging ratio don't reach 50% at the concentration of 0.5mM (see Table 3 note). The results prove that morin hydrate containing two hydroxyl in ring B has better $O_2^{\bullet-}$ scavenging activity than hydroxysafflor yellow A which only has one hydroxyl in ring B (see Fig.5). Some methods have been used to evaluate the antioxidant activities of curcumin involving superoxide scavenging ability[22] due to its phenolic hydroxyl groups, or the methylene group of β-diketone moiety, or the benzylic hydrogens[23-24].The results show that the IC_{50} value of curcumin is 0.244mM which is roughly equal to *L*-ascorbic acid (0.219mM). So curcumin has strong $O_2^{\bullet-}$ scavenging activity. Carotenoids, to be occurred in vegetables and fruits, have been associated with reduced risk of degenerative diseases. It's well known that lutein and β-carotene contribute significantly to antioxidant activity[16]. The lower IC_{50} value of lutein in Table 3 indicates that lutein has higher $O_2^{\bullet-}$scavenging activity than that of *L*-ascorbic acid. β-Carotene, by contrast, has the lower $O_2^{\bullet-}$ scavenging activity. Miller et al. [15]

reported that the increasing of the ROS scavenging activities of carotenoids depend on the conjugated double-bond system and the presence of function groups like hydroxyl groups. Although lutein has similar conjugated double-bond structure with that of β-carotene, it has greater antioxidant activity than that of β-carotene. This result may be from the hydroxyl groups of 3- and 3'-positions in the structure of lutein. Crocin Ⅰ and Ⅱ are two of a few water soluble carotenoids found in nature.Therefore, they have been used widly as a food colorant or antioxidant[25-26]. Compared with lutein, the different conjugated double-bond of crocins might be the reason of their lower antioxidant activities.

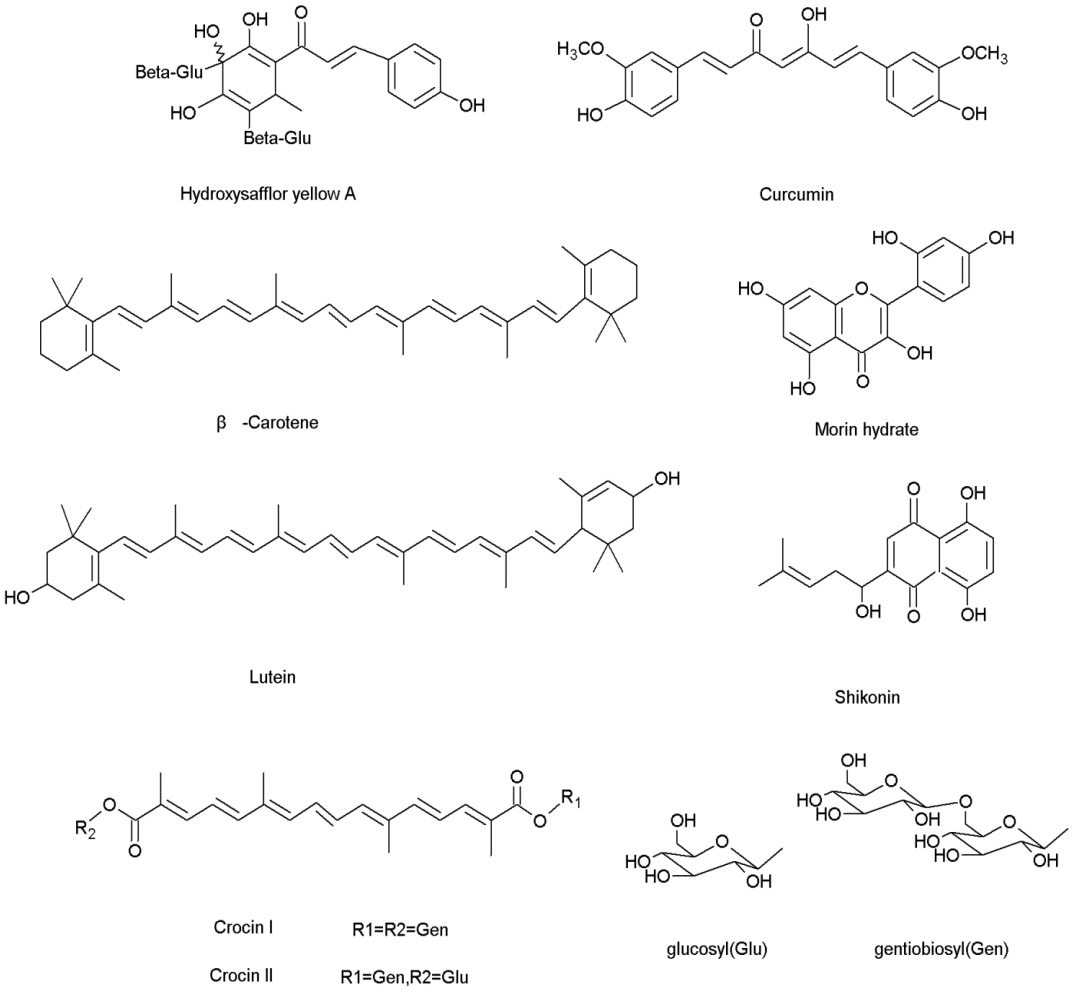


Fig.5 Chemical structures of the evaluated natural pigments by UPLC-DAD method

CONCLUSIONS

We have established a simple and rapid method of UPLC-DAD for measuring $O_2^{\bullet-}$ scavenging activity. As we know that many natural pigments, such as lutein and curcumin, have strong antioxidant activities. However, there was no appropriate method for testing $O_2^{\bullet-}$ scavenging activities of these pigments. Compared with traditional methods, as a new probe, WST-1 was highly water-soluble, thus the problem of water-insolubility of NBT formazan in NBT method as well as the low sensitivity of cytochrome c method and so forth were solved. In this study, we successfully measured $O_2^{\bullet-}$ scavenging activities of nine natural pigments under the optimized conditions, and the results are consistent with the previous studies[27-29]. Even more importantly, the method could be applied to those samples with strong absorptions in the visible region, especially for natural pigments, in which case, UV-Vis spectrophotometry method could not be used. In addition, the UPLC-DAD method could be also used for screening other superoxide anion radical scavengers.

Acknowledgements

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