Analytical Methods

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Journal:	*Analytical Methods*
Manuscript ID:	AY-ART-03-2015-000614.R1
Article Type:	Paper
Date Submitted by the Author:	14-Apr-2015

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A high efficient and accurate method for finding antioxidant chemicals from *Capsicum annuum L.*

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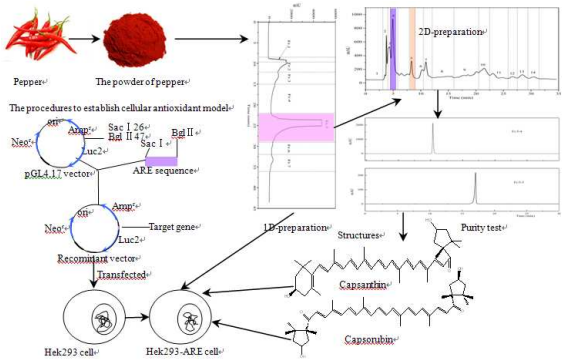


A novel cellular antioxidant activity model based on Hek293 cells was established to evaluate the antioxidant ability by the expression level of the luciferase reporter gene.

Abstract

A two-dimensional preparative HPLC used in non-aqueous mobile phase was constructed for the separation of weak polar compounds from *Capsicum annuum L.* As a result, Silica column coupled with Amide column in non-aqueous mobile phase showed good orthogonality. Two single compounds with the purity of more than 97%

were obtained efficiently. Then a unique cellular antioxidant activity (CAA) model by using anti-oxidative response element (ARE) to regulate the expression level of luciferase reporter gene was employed to evaluate the relative antioxidant activities of purified compounds with Tertiary Butyl Hydroquinone (TBHQ) as the positive control. The unit (LCAA) for the measurement of the antioxidant capacity was defined. The antioxidant capacity of 1.5 μg/mL TBHQ was defined as 1 LCAA. At last, the relative antioxidant capacities of the two active compounds Fraction 5-4 and Fraction 5-5 were 0.397 LCAA and 0.523 LCAA respectively. The protocols used above will greatly promote the research of functional food.

Keywords: Non-aqueous HPLC; two-dimensional preparation; anti-oxidative response element; luciferase activity; cellular antioxidant activity.

Introduction

Red pepper (*Capsicum annuum L.*) is an important vegetable widely cultivated and used throughout the world. The red color of red pepper is due to the various carotenoid pigments including oxygenated carotenoids. These compounds showed antioxidant activity, stimulating the immune system, preventing cardiovascular diseases, and delaying the aging process[1]. Previous studies have demonstrated the effectiveness of anti-oxidative components in pepper[2]. Red pepper is so widely used in the dinner of thousands of people every day. It is very

desirable to establish an efficient method for the further research of red pepper.

Preparative HPLC, one of the most efficient technologies applied in the preparation of single chemicals in complex samples, takes the advantages of high performance separation, on-line monitoring and automatic control to realize the efficient preparation of single compounds[3]. Its application in separating compounds from complex red pepper attracts more and more attention[4]. However, taking the complex chemical composition of the nature medicine extracts into account, we think it is virtually impossible to obtain compounds of high purity by only one dimensional preparation due to the limited resolution and peak capacity.

Two-dimensional HPLC has become a powerful tool to separate compounds from complex samples, since it has made great improvement in separation selectivity and peak capacity[5]. In most of two dimensional HPLC preparation methods[3,6,7], water is usually taken as one of the mobile phase because RPLC is so widely used. For the small weak polar chemicals in the red pepper that are hardly dissolved in aqueous solution, thus, mobile phases containing water seem to be improper. A high-efficiency two-dimensional HPLC separation method for complex weak polar food extracts by HPLC preparative columns used in non-aqueous mobile phase is quite needed.

In the search for natural antioxidants from plant sources, numerous studies on the antioxidants derived from fruits and vegetables have revealed that ascorbic acid, carotenoids, polyphenols, and tocopherols have high antioxidant activities[8]. Increasing experimental evidence has suggested that these compounds provide protection against harmful free radicals on cellular components such as DNA, proteins, and lipids, and have been associated with lower incidence and mortality rates of cancer and cardiovascular diseases, in addition to a number of other health benefits[9, 10].

Several assays have been routinely used to estimate the antioxidant ability of natural products. 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay[11], ferric reducing antioxidant power (FRAP) assay[12], oxygen radical absorption capacity (ORAC) assay[13, 14], β-carotene bleaching assay[15] and 2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) assay[16] are the most common antioxidant activity test methods in the research of functional food. However, these assays are simple chemical methods without taking cell physiological environment characters into account. In fact, biological systems are much more complex than the simple chemical mixtures mentioned above. The most accurate method evaluating the antioxidant capacities of compounds is using *in vivo* experiments on animal model or human bodies. However, these *in vivo* assays are expensive and time-consuming, which are not suitable for initial activity screening. Therefore, a novel cellular antioxidant activity model based on normal human embryonic kidney cells was established by molecular biology technology in our laboratory. Compared with the chemical methods above, this method can greatly reduce the false positive results of antioxidant activity screening experiments.

In this article, we used the 2D-prep-HPLC and the new CAA assay together to evaluate the responsible chemicals for the antioxidant activity of red pepper. These efficient protocols were suitable for most of the weak polar chemical compounds research in functional food extracts, and *Capsicum annuum L.* weak polar crude extract was taken as an example to explain it.

Results and discussion

Chromatographic work

The first dimensional preparation

The first dimensional preparation was performed on a Silica prep column. The sample loading was about 200 mg per injection. One whole preparation procedure took 75 minutes which consisted of 15 minutes for column balance, 15 minutes for column washing and 45 minutes for preparation. Because of the complex composition of the crude sample, the retention time of the components differed a lot. A simple isocratic method was not enough. Gradient elution was necessary for the first dimensional preparation. Linear gradient offered the best separation performance for the components with quite different retention time. Finally, elution method 0-45 minutes for 5% to 10% B was used for the preparation. Preparation of the total 5.2 g crude sample solution took 26 injections in 40 hours. The fractions were collected according to the UV absorption intensity to reduce the complexity of each fraction as much as possible. As shown in Fig. S1 in supplementary information, the cross in each fraction had been minimized because of the good separation repeatability. As shown in Fig. 1, 7 fractions were collected in the first dimensional separation and their amounts by weight were at the range from 0.1 g for Fraction 2 to 2.9 g for Fraction 5, and the weight of the rest fractions were about 0.2 g. The recovery at YMC Silica column was about 76%.

Stationary phase selection

In the fractions separated from the first dimensional separation, only Fraction 5 had some antioxidant activity. So the second dimensional separation was focused on Fraction 5. The separation capacity of four HPLC columns with different stationary phases (Fig. S2 in supplementary information) was tested in this section to select the best one for the following second dimensional preparation. Fig. 2 showed that the elution order and the retention time of the peaks in different column chromatograms were quite different from Silica column used in the first dimensional separation, which indicated that one of the four columns was possible to construct an orthogonal two dimensional separation system. It was demonstrated from Fig. 2 (c) and (d) that the retention capacity of the Diol and Maltose column was good when separating Fraction 5. However, the selection characters of these two columns were not satisfying that the main chemicals were still in a big chromatography peak at about 10 min with similar retention factors instead of being separated from each other with high enough resolution. These two columns above were not suitable for purification of the compounds in Fraction 5. When we came to Fig. 2 (b), it was easily found that the retention factors of the compounds on CN column were so small that they were hardly be separated, due to the too mild CN column stationary phase polarity compared with that of bare silica. The CN column was not suitable for the second separation either. Moving to Fig. 2 (a) produced by Amide column, it was found that the main chemicals were separated from each other with high enough resolution, which were recognized as single

peaks. In fact, the polarity of the stationary phase of the Amide column used under non-aqueous mobile phase condition was higher than CN column but lower than Diol and Maltose columns. We thought its medium polarity was part of the reason why it was the right one, which provided appropriate retention capacity to separate compounds in a retention time range 5 to 10 min.

Preparation of the single compounds

In order to purify the compounds effectively, the separation condition was optimized for each fraction. In this method, the optimization conditions were performed on an analytical scale. The analytical scale conditions were transformed to preparative scale conditions. It was found that isocratic conditions were enough for the separation of all the fractions, and the application of an isocratic elution method would avoid extra time for reconditioning in gradient elution. The content of ethanol was optimized to ensure the retention time of the peaks between 5 and 20 minutes. After a minor revision of the elution method of the analytical conditions, the separation condition was easily transformed to a preparative scale, as shown in Fig. 3. The analytical chromatograms in Fig. 2, showed similar patterns with the preparative chromatograms in Fig. 3, which demonstrated the feasibility of the transformation from an analytical scale to a preparative one. In addition, it was noteworthy that heart-cutting was used as the repeated separation strategy to insure the purity of compounds. The preparation chromatograms of Fraction 5 were shown in Fig. 3.

After isolating Fraction 5, two pure compounds were obtained and dried by rotary evaporation at 60 °C in a vacuum. All in all, compound 1 (Fraction 5-4, 7.37 mg) and 2 (Fraction 5-5, 3.21 mg) had enough amounts to be characterized by NMR and MS. In consideration of the impurities with quite different retention times, it was not appropriate to conduct the homogeneity test with isocratic elution methods. The content of the strong elution mobile phase was not higher than 3% in the second dimensional preparation. The purities of these compounds checked by HPLC were shown in Fig. 4 and the purities of all compounds were more than 97%.

Compared with previous reverse phase liquid chromatography for separation of *capsanthin* and *capsorubin* with aqueous mobile phase[17], non-aqueous mobile phase used in our separation protocols could increase the solubility of carotenoids, which could increase the loading capacity and improve chromatographic efficiency and save much time. What's more, compared with the previous similar methods[18], our novel separation method increased the retention time of *capsanthi*n and *capsorubin* and improved peak shade, which eventually increased the resolution of the column. Additionally, compared with traditional column chromatography needing a lot of manual operation, our 2D-HPLC purification method, benefiting from automatic on-line control, greatly improved the efficiency of separation work.

Cellular antioxidant activity assay

Quantification of CAA assay

To be able to compare data in the literature from different laboratories, the CAA method should be standardized. On the basis of our results, we strongly recommend that positive control TBHQ was used as a standard in this new assay for quantifying cellular antioxidant activity for the following reasons: (a) TBHQ had high CAA activity compared to other chemicals; (b) the pure compound was easily and economically obtained; and (c) it was relatively stable. In the experiment, in fact, TBHQ was greatly linear related to its luciferase activity in the concentration range 0.5-6.0 μg/mL (related coefficient was 0.9962):

$$Y = (X - 279)/6332$$

Among which, X was the luciferase activity of positive TBHQ and Y was the corresponding TBHQ concentration. In each experiment, TBHQ was used as a standard, and cellular antioxidant activities for pure compounds were expressed as micromoles of TBHQ equivalents (TE) per 1.5 μg/mL of tested compound. To make the method handier, we defined a unit (LCAA) for the measurement of the relative antioxidant capacity. The antioxidant capacity of 1.5 μg/mL TBHQ was defined as 1 LCAA and the relative antioxidant capacity of the compound was defined as:

$$\text{Antioxidant capacity(LCAA)} = \left(\frac{X - 279}{6332}\right) \Big/ LA_{1.5}$$

Among which, X was the luciferase activity of compound tested in CAA assay. $LA_{1.5}$ was the concentration of TBHQ (1.5 μg/mL). The results of the relative antioxidant capacities of the fractions from the first dimensional separation were shown in Table 1 that Fraction 5 showed the best relative antioxidant activity. Then Fraction 5 was further separated on YMC Amide column to obtain single compounds. As shown in Table 1, the relative antioxidant capacities of the two active compounds Fraction 5-4 and Fraction 5-5 were 0.397 LCAA and 0.523 LCAA respectively.

Advantages of CAA Assay

Antioxidant effects exerted by plant compounds were often attributed to their enhancement of antioxidant defense systems. These processes were mostly mediated by *in vivo* human detoxifying/antioxidant enzymes. The induction of these enzymes was attributed to the NF-E2-related factor-2 (Nrf2) which binds to an upstream regulatory sequence (ARE) and activates the expression of various genes encoding cytoprotective proteins[19]. TBHQ could act as a good *in vivo* positive control because it was a strong activator of Nrf2, which could indirectly activate ARE to resist oxidation. The traditional chemical antioxidant assays[11-13] had the limitation of the inability to represent the complexity of biological systems. They measured chemical reactions only, and these reactions could not be interpreted to represent activity *in vivo*, as they could not account for the bioavailability, stability, tissue retention, or reactivity of the compounds under physiological conditions.

The points above could also be clearly explained by the comparing of how TBHQ was used in traditional

chemical antioxidant method and in our CAA assay. The phenolic structure of TBHQ caught a free radical (O·) and let the free radical make itself oxidized for the purpose of eliminating free radical or anti-oxidation[20]. However, the anti-oxidative mechanism of traditional chemical assay was totally different with that of human body. So it was very easy to obtain false positive results from traditional chemical anti-oxidative screening assay. Our CAA assay shared the same mechanism as actual human *in vivo* Nrf2-ARE antioxidant pathway. It used a reporter gene "Luc" to test whether the compounds could activate ARE. Compared with the traditional chemical anti-oxidative screening assays, it could give researchers much more credible antioxidant data.

Structure identification

Single compounds separated from *Capsicum annuum L.* were identified by NMR and MS. Compounds Fraction 5-4 and Fraction 5-5 were identified as *capsanthin* and *capsorubin*, with the characters as follows: the structure of Fraction 5-4 from *Capsicum annuum L.* was identified by MS and ^{13}C NMR. FAB-MS *m/z* 584 $[M]^+$. ^{13}CNMR: δ=12.7 (C17′, CH3), 12.8 (C16′, C17, C19, C20, CH3), 12.9 (C16, CH2), 21.6 (C18, CH3), 25.1 (C20′, CH3), 25.4 (C18′, CH3), 25.9 (C19′, CH3), 37.1 (C1, C), 42.6 (C4, CH2), 44.0 (C1′,C), 45.3 (C4′, CH2), 48.5 (C2, CH2), 51.0 (C2′, CH2), 59.0 (C5′, C), 65.1 (C3, CH), 70.4 (C3′, CH), 121.0 (C7′, CH), 124.1 (C11′, CH), 125.5 (C11, CH), 125.9 (C7, CH), 126.1 (C5, C), 129.7 (C15, CH), 131.3 (C15′, CH), 131.7 (C10, CH), 132.4 (C14, CH), 135.3 (C14′, CH), 136.1 (C13′, C), 136.6 (C9′, C), 137.4 (C12, CH), 137.6 (C9, C13, C), 137.8 (C6, C), 138.5 (C8, CH), 140.7 (C10′, CH), 142.0 (C12′, CH), 146.9 (C8′, CH), 202.9 (C6′,C). Compared with previous literature[21, 22], it was identified as *capsanthin*.

The structure of Fraction 5-5 was identified by MS and ^{13}C NMR. FAB-MS *m/z* 600 $[M]^+$.^{13}C NMR: δ=12.8 (C19, C20, C19′, C20′, CH3), 25.1 (C16, C17, C16′, C17′, CH3), 25.9 (C18, C18′, CH3), 44.0 (C1, C1′, C), 45.3 (C4, C4′, CH2), 50.8 (C2, C2′, CH2), 59.0 (C5, C5′, CH3), 70.3 (C3, C3′, CH), 121.1 (C7, C7′, CH), 124.7(C11, C11′, CH), 131.2 (C15, C15′, CH), 134.0（C9, C9′, C）, 134.9 (C14, C14′, CH), 136.9 (C13, C13′, C), 140.6 (C10, C10′, CH), 141.8 (C12, C12′, CH), 146.8 (C8, C8′, CH), 203.0 (C6, C6′, CH). Compared with previous literature[21, 23], it was identified as *capsorubin*.

Experimental

Apparatus

The semi-preparation was carried on Agela CHEETAH HP 100 system. The system included a UV detector, two binary gradient pumps, a sample loop and Agela LC software (CHEETAH HP 100; Bonna-Agela Technologies Inc,Tianjin,China).

Chromatographic analysis was performed on a Hitachi HPLC system which consisted of a Hitachi L2400 UV detector, a Hitachi L2130 pump, a Hitachi L2200 autosampler and Hitachi Lachrom Elite HPLC software (L2000; Hitachi Ltd., Tokyo, Japan).

The structures of compounds were identified by MS and NMR. Mass spectrometry was performed on a Varian Saturn 2200 (Saturn 2200; VARIAN medical systems, Salt Lake, USA). The NMR spectrum was measured on a Bruker 600 NMR spectrometer with CD3OD as solvent (Bruker AVANCE III; Bruker Corporation, Karlsruhe, Germany).

Inverted phase contrast fluorescence microscope was purchased from Japan Nikon Company (Ti-U; Nikon Corporation, Tokyo, Japan). CO2 cell incubator was obtained from RS Biotech (Galaxy S; RS Biotech, Scotland, England). Cell culture plates and bottles were purchased from Corning (430372 and 3596; Glendale, America). Biological safety cabinets were received from Haier Company (HR40-IIA2; Qingdao, Shandong Province, China).

Reagents

Industrial and prep-HPLC grade ethanol, n-hexane and ethyl acetate were purchased from Concord Technology Co. Ltd (Tianjin, China). Tertiary butylhydroquinone (TBHQ) and Agarose gel were purchased from Sigma-Aldrich Corporation (St. Louis, USA). Dimethyl Sulfoxide (DMSO) and resistance screening reagent Geneticin (G418) were obtained from Solarbio Science and Technology Co.,Ltd (Beijing, China). Plasmid extraction and purification kit, T4 DNA ligase and related reagents were purchased from Toyobo Life Science Department (Osaka, Japan). DNA ladder maker was purchased from Fermentas (MBI). Doublecco's modified eagle's medium (DEME) was obtained from Thermo Electron Corporation (Massachusetts, USA). Trypsin was acquired from Invitrogen Company (California Prefecture, Carlsbad City, USA). Human embryonic kidney epithelial cells (Hek293 cells) were cultured by our laboratory (Tianjin, China). FuGENE® HD Transfection Reagent was got from Roche (Basel, Switzerland). Steady-Glo™ Luciferase Assay System was Promega Corporation (Madison, USA).

Sample preparation

Capsicum annuum L. was purchased from Chenguang Biotech Group (Handan, Hebei Province, China) and authenticated by Professor Mei Lijuan, Northwest institute of plateau biology, Chinese Academy of Science. One kilogram of *Capsicum annuum L.* was powdered by a *Cordyceps sinensis* ultrafine grinder machine (SQW-25; San Qing Technology Company, Jinan, Shandong Province, China) under minus forty degree Celsius to protect the chemicals in the materials from degradation. Then the superfine powder was extracted by 40 L 95% ethanol at 60 °C for 180 min three times. The ethanol extract of *Capsicum annuum L.* was centrifuged. The supernatant was then combined and concentrated by rotary evaporation (RE-52AA; Shanghai Yarong biochemical instrument factory, Shanghai, China) at 60 °C in a vacuum. The concentrated extract was re-dissolved in solution of n-hexane/ethanol 97:3 v/v and kept for 24 hours at 4 °C. After centrifugation, the supernatant was filtered through 0.45 μm membranes. The final concentration of the filtered sample solution was 42.7 mg/mL.

Chromatography condition

The firstdimensional preparation was performed on Silica column (50 mm × 250 mm i.d., 10 μm, 100 Å, YMC). N-hexane was mobile phase A and ethanol was mobile phase B. The linear elution protocol was 0-45 min

5% to 10% B. The injection volume was 5 mL at the flow rate of 80 mL/min. The UV detection was recorded at 260 nm.

The fraction (Fraction 5) with the best antioxidant activity was then analyzed on other four different analytical columns to select the best stationary phase to run the second dimensional separation, including Amide (4.6 mm × 250 mm i.d., 5 μm, 100 Å, YMC) column, Maltose (4.6 mm × 250 mm i.d., 5 μm, 100 Å, YMC) column, CN (4.6 mm × 250 mm i.d., 10 μm, 120 Å, YMC) column and Diol (4.6 mm × 250 mm i.d., 10 μm, 60 Å, Kromasil) column. The mobile phase A was n-hexane and B was ethanol. The isocratic elution procedures on the columns were the same. The isocratic elution procedure was 96% A for 35 min. Chromatographic data was collected at 260 nm. At last, Amide stationary phase was proved to have the best selectivity and peak resolution. So the preparative Amide column (20 mm × 250 mm i.d., 10 μm, 100 Å, YMC) was chosen as the second dimensional separation column. After some minor revision on the base of the elution method used for analytical Amide column, the isocratic elution procedure 97% A for 35 min was employed for the second dimensional separation. The injection volume was 2 mL and the flow rate was 16 mL/min.

Purity test of the purified single compounds was carried on Amide analytical column (4.6 mm × 250 mm i.d., 5 μm, 100 Å, YMC). N-hexane was mobile phase A and ethanol was mobile phase B. The injection volume was 10 μL. The linear gradient elution procedure was 1% to 3.5% B for 30 min. The flow rate was 1 mL/min. The detection wavelength was 260 nm.

Cellular antioxidant activity assay

Construction of Luc-CAA cell model

Hek293 cells were maintained in Dulbecco's modified Eagle's medium, and placed at 37 °C with 5% carbon dioxide; media were changed every 3-4 days. A 219 bp clone fraction containing ARE sequence (5'-CTCAGCCTTCCAAATCGCAGTCACAGTGACTCAGCAGAA-3') was used. The ARE has 5 repeats, which was connected by sequence "CCC". The DNA segment was excised with Sac I and Bgl II, and subcloned into pGL4.17. The cells were plated in 6-well plates, incubated overnight, and transfected with pARE-Luc-Neo using FuGENE® HD Transfection Reagent. Forty-eight hours after transfection, the cell culture media were replaced with fresh media, containing resistance screening reagent G418 at a final concentration of 800 mg/L. The media were changed every 3-4 days. Cell clones were then selected by limiting dilution in the presence of G418 to generate stable lines. Clones that were resistant to G418 were selected to identify clones that were positive for expression of luciferase (Luc) and presence of the ARE DNA segment by PCR. To test for inducible Luc expression, clones were incubated with 25 μm/L TBHQ. Luc activity was measured using the Steady-Glo™ Luciferase Assay System per the manufacturer's instructions. The clones with the highest Luc activity were used as screening model with the name of Hek293-ARE. Negative cell clones were generated by transfecting pGL4.17 as described above.

Activity screening by Luc assay

Luc activity was measured using the Steady-Glo™ Luciferase Assay System per the manufacturer's instructions. Briefly, stably transfected cells were used to seed in 96-well clear bottom white plates (3596) 24 h before treatment. After stimulation with compounds for 24 h, the cells were lysed directly in Steady-Glo™ Luciferase Assay System reagent and mixed on a shaker for 2 min at room temperature. The Luc activity of the cell lysates was measured on a Multi Reader (Varioskan flash; Thermo Fisher SCIENTIFIC, Waltham, USA). Luc activity was normalized to cell number by protein concentration by BCA assay (Pierce, Waltham, USA).

Conclusions

On the basis of the new 2D-prep-HPLC and novel CAA assay constructed in this article, two single compounds capsanthin and capsorubin were separated and identified from the weak polar extract of red pepper. The purities of the compounds were more than 97%. And the relative antioxidant activity values of capsanthin and capsorubin were 0.397 LCAA and 0.523 LCAA respectively. The novel 2D-prep-HPLC method here not only provided full set weak polar sample pretreatment methods but also a non-aqueous HPLC separation system with high orthogonality, which could make high purity weak polar compounds preparation much more efficient. What's more, the activity screen method used in this article based on Keap1-Nrf2-ARE signal pathway shared the same mechanism as actual human *in vivo* antioxidant pathway. It could give much more credible antioxidant results compared with traditional chemical antioxidant assay. We believe this 2D-prep-HPLC and novel CAA assay will significantly do some favor for the functional food research by finding more useful active compounds to meet the demands of the functional food market.

Acknowledgements

This work was supported by National High Technology Research and Development Program 863 (2011AA100603).

Bibliographic references and notes

1 N. I. Krinsky and E. J. Johnson, *Mol. Aspects Med.*, 2005, **26**, 459-516.

2 A. M. Chuah, Y. C. Lee, T. Yamaguchi, H. Takamura, L. J. Yin and T. Matoba, *Food Chem.*, 2008, **111**, 20-28.

3 J. Feng, Y. Xiao, Z. Guo, D. Yu, Y. Jin and X. Liang, *J. Sep. Sci.*, 2011, **34**, 299-307.

4 J. Deli, Z. Matus, P. Molnár and G. Tóth, *Eur. Food Res. Technol.*, 2001, **213**, 301-305.

5 M. Vollmer, P. Hörth and E. Nägele, *Anal. Chem.,* 2004, **76**, 5180-5185.

6 Q. Fu, Z. Guo, X. Zhang, Y. Liu and X. Liang, *J. Sep. Sci.*, 2012, **35**, 1821-1827.
7 E. Nägele, M. Vollmer and P. Hörth, *J. Chromatogr. A*, 2003, **1009**, 197-205.
8 C. Kaur and H. C. Kapoor, *Int. J. Food Sci. Tech.*, 2001, **36**, 703-725.
9 Y. S. Velioglu, G. Mazza, L. Gao and B. D. Oomah, *J. Agric. Food Chem.*, 1998, **46**, 4113-4117.
10 H. Wang, G. Cao and R. L. Prior, *J. Agric. Food Chem.*, 1996, **44**, 701-705.
11 M. S. Blois, *Nature*, 1958, **181**, 1199-1200.
12 I. F. F. Benzie and J. J. Strain, *Anal. Biochem.*, 1996, **239**, 70-76.
13 G. Cao, H. M. Alessio and R. G. Cutler, *Free Radical Bio. Med.*, 1993, **14**, 303-311.
14 A. N. Glazer, *Method. Enzymol.*, 1990, **186**, 161-168.
15 F. Menichini, R. Tundis, M. Bonesi, M. R. Loizzo, F. Conforti, G. Statti, B. De Cindio, P. J. Houghton and F. Menichini, *Food Chem.*, 2009, **114**, 553-560.
16 R. van der Werf, C. Marcic, A. Khalil, S. Sigrist and E. Marchioni, *LWT - Food Sci. Technol.*, 2014, **58**, 77-85.
17 P. A. Biacs, H. G. Daood, A. Pavisa and F. Hajdu, *J. Agr. Food Chem.*, 1989, **37**, 350-353.
18 M. Weissenberg, I. Schaeffler, E. Menagem, M. Barzilai and A. Levy, *J. Chromatogr. A*, 1997, **757**, 89-95.
19 G. Shen, W. S. Jeong, R. Hu and A. N. T. Kong, *Antioxid. Redox Sign.*, 2005, **7**, 1648-1663.
20 L. S. de Sousa, C. V. R. de Moura, J. E. de Oliveira and E. M. de Moura, *Fuel*, 2014, **134**, 420-428.
21 T. Maoka, Y. Goto, K. Isobe, Y. Fujiwara, K. Hashimoto and K. Mochida, *J. Oleo Sci.*, 2001, **50**, 663-665.
22 M. Baranyai, J. Szabolcs, G. Tóth and L. Radics, *Tetrahedron*, 1976, **32**, 867-870.
23 G. Moss, *Pure App. Chem.*, 1976, **47**, 97-102.

Figure captions

Fig. 1 The first dimensional preparation for crude sample on Silica column (50 mm × 250 mm i.d., 10 μm, 100 Å, YMC). Mobile phase: (A) n-hexane (B) ethanol; he linear elution 5% to 10% B for 45 min; the injection volume 5 mL; flow rate 80 mL/min; UV detection: 260 nm.

Fig. 2 The analytical chromatograms of Fraction 5 on four different columns. (a) Amide column; (b) CN column; (c) Diol column; (d) Maltose column. Mobile phase: (A) n-hexane (B) ethanol; isocratic elution 97% A for 35 min; flow rate: 1 mL/min; UV detection: 260 nm.

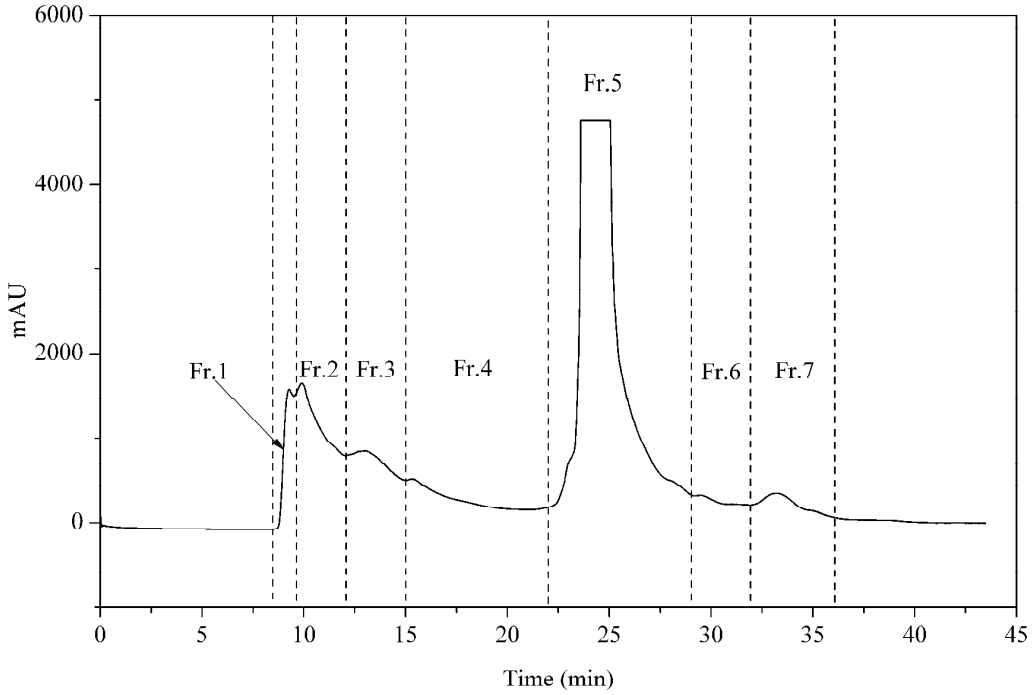
Fig. 3 Second dimensional preparation of Fraction 5 on preparative Amide column (20 mm × 250 mm i.d., 10 μm, 100 Å, YMC). Mobile phase: (A) n-hexane (B) ethanol; the isocratic elution 97% A for 35 min; the injection volume 2 mL; flow rate 16 mL/min; UV detection: 260.

Fig. 4 Purity test of Fraction 5-4 and Fraction 5-5 on Amide column (4.6 mm × 250 mm i.d., 5 μm, 100 Å, YMC). Mobile phase: (A) n-hexane (B) ethanol; the gradient elution: 1-3.5 B for 35 min; the injection volume 10 μL; flow rate 1 mL/min; UV detection: 260 nm.

Fig. S1 The repeatability of the first dimensional preparation. The repeatability is presented by comparing the chromatography of the 1st, 5th, 10th and 15th injection.**Fig. S2** The stationary phases for four columns. (a) Amide column; (b) CN column; (c) Diol column; (d) Maltose column.

Table 1 The relative antioxidant capacity of the compounds.

Sample	The relative antioxidant capacity (LCAA)
Control (TBHQ)	1.000
Fraction 1	0.176±0.032
Fraction 2	0.388±0.035
Fraction 3	0.010±0.042
Fraction 4	0.041±0.055
Fraction 5	0.764±0.047
Fraction 6	0.208±0.031
Fraction 7	0.317±0.039
Fraction 5-4	0.397±0.026
Fraction 5-5	0.523±0.045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Fr.2
Fr.3
Fr.4
Fr.5
Fr.6
Fr.7
0
5
10
15
20
25
30
35
40
45
Time (min)

Fig. 1

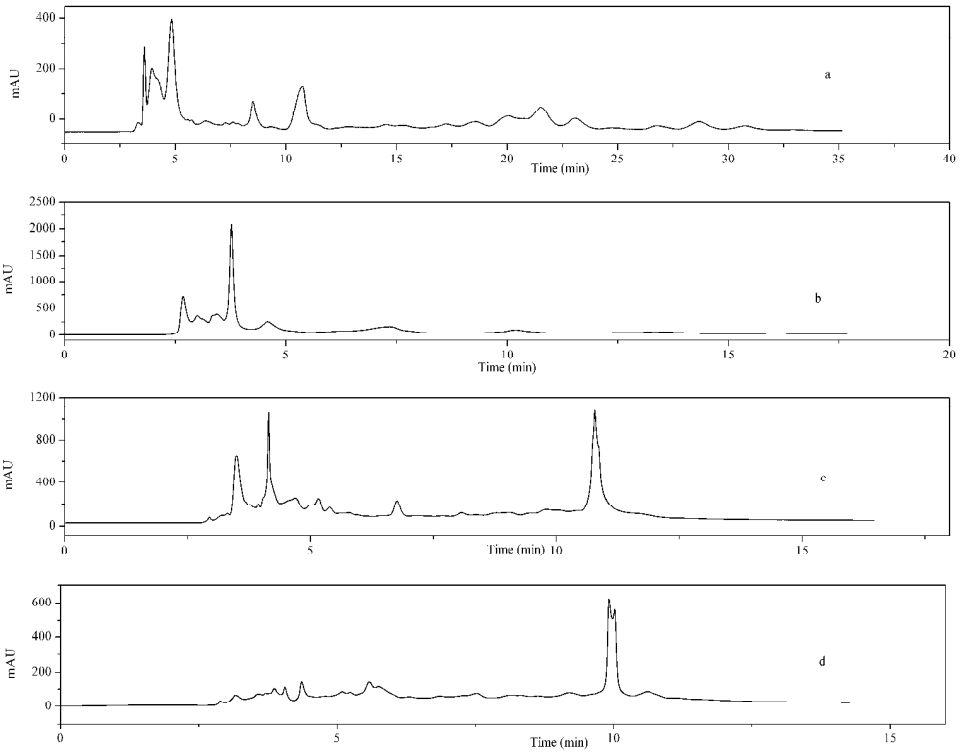

Fig. 2

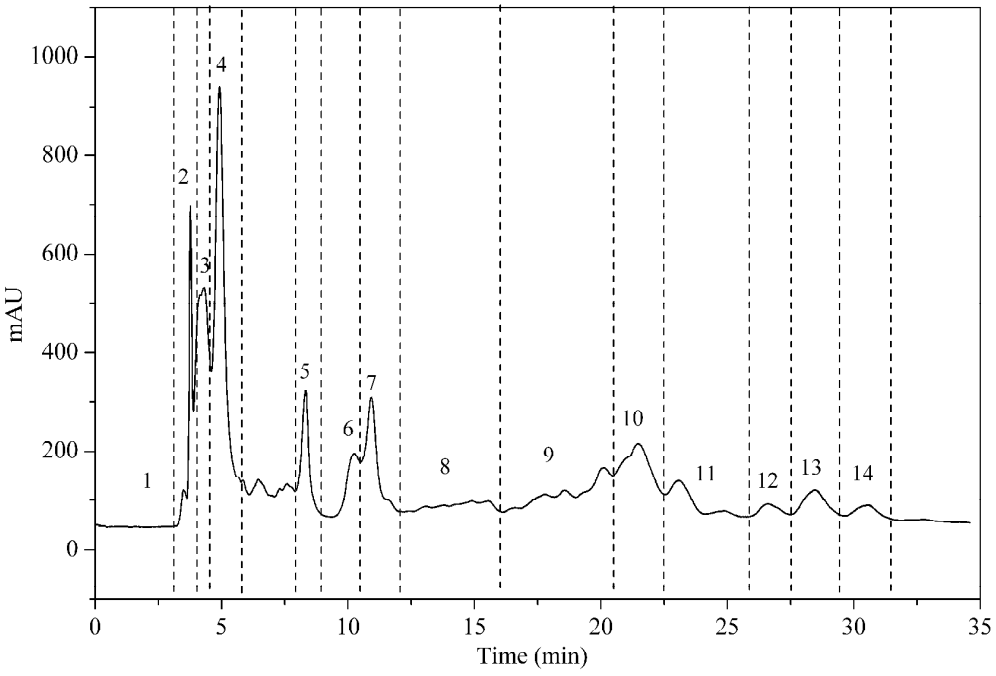
1000
800
600
400
200
0
mAU
0
5
10
15
20
25
30
35
Time (min)
1
2
3
4
5
6
7
8
9
10
11
12
13
14

Fig. 3

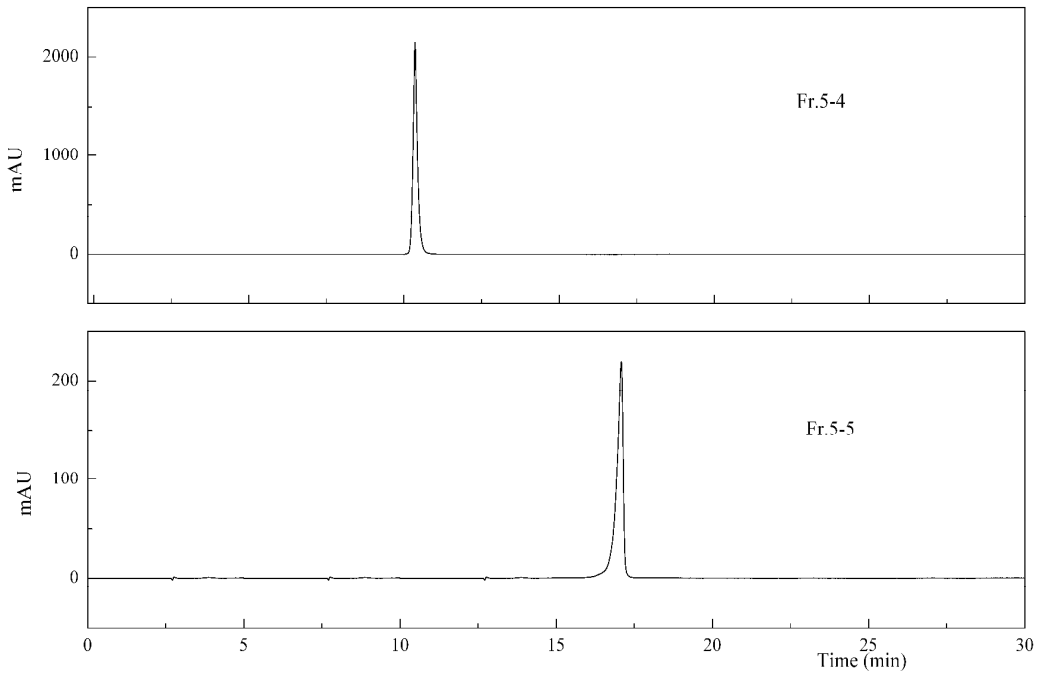

Fig. 4