Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1048 (2004) 17–24
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

An improved method for the analysis of major antioxidants of *Hibiscus esculentus* Linn

Guanghou Shui, Leong Lai Peng*

Department of Chemistry, Food Science and Technology Programme, National University of Singapore, 3 Science Drive 3, Singapore 117543, Singapore

Received 25 February 2004; received in revised form 15 July 2004; accepted 15 July 2004

Abstract

Major antioxidants of aqueous ethanol extract from Lady's Finger (*Hibiscus esculentus* Linn) were systematically investigated in this study. Firstly, high-performance liquid chromatography (HPLC) was applied to identify antioxidant peaks in a sample by spiking the sample extract with 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) free radical, which was prepared from manganese dioxide and ABTS. Secondly, in order to identify the elution period of major antioxidant peaks, the antioxidant capacities of different fractions from solid-phase extraction (SPE) were measured, and the chromatograms of fractions were also recorded. Lastly, multiple mass spectrometry (MS^n) was used to elucidate the possible chemical structures of antioxidants, and nuclear magnetic resonance (NMR) was further applied for structure confirmation. The major antioxidant compounds in lady's finger were identified to be quercetin derivatives and (−)-epigallocatechin using HPLC–MS and HPLC–MS^n (n = 2–4) techniques. It was found that about 70% of total antioxidant activity was contributed by four quercetin derivatives. The structures of major antioxidants, which were isolated by semi-preparative RP-HPLC with two tandem C_{18} columns, were further confirmed using UV–vis absorption spectroscopy and ^{13}C NMR spectra. Quercetin 3-*O*-xylosyl (1‴ → 2″) glucoside, quercetin 3-*O*-glucosyl (1‴ → 6″) glucoside, quercetin 3-*O*-glucoside and quercetin 3-*O*-(6″-*O*-malonyl)-glucoside were first identified and characterized as major antioxidants in lady's finger.

Keywords: Antioxidants; Solid phase extraction; Lady's finger; Flavonoids

1. Introduction

A free radical ($F^{\bullet}$) is any species capable of independent existence containing one or more unpaired electrons [1]. Most free radicals are unstable and thus highly reactive since they need to pair their unpaired electrons. Radicals are produced by normal aerobic metabolism and are necessary to life. Our immune system needs free radicals to fight invading bacteria and viruses. However, excess amount of free radicals are harmful because they will damage DNA, protein and lipids, which are the most important biomolecules in human body. Their oxidative damages are discussed as pathobiochemical mechanisms involved in the initiation or progression phase of various diseases [2,3]. Scientists increasingly believe they play a major role in the development of many aging-related diseases, like cancer, cataract and heart diseases. Therefore, it is very important to keep the content of free radicals to a certain lower value. It is believed that antioxidant protects our bodies from free radical damages and thus plays a central role in prevention of aging-related diseases.

It has long been recognized that fruits and vegetables are essential to a healthy and well balanced diet required for healthy living. It has also been recognized that high consumption of some fruits and vegetables is beneficial to health in combating the onset of cancer, coronary diseases, inflammation, arthritis, immune system decline, brain dysfunction and cataracts [4–9]. These protective effects are considered, in large part, to be related to the various antioxidants contained in them which are responsible for the scavenging of reactive

* Corresponding author. Tel.: +65 68742917; fax: +65 67757895.
E-mail address: laipeng@nus.edu.sg (L.L. Peng).

0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2004.07.032

free radicals mentioned above. There are many different antioxidant components with different physical and chemical properties in fruits and vegetables, thus it is relatively difficult to measure each antioxidant component separately. As reviewed by Prior and Cao, a number of methods were established to evaluate the overall antioxidant capacity or total antioxidant activity (TAA) of fruits and vegetables [10]. However, from the TAA value of a fruit/vegetable one will not be able to obtain information about which compounds are major antioxidants in the mixture. In addition, the knowledge of the potential antioxidant components present in fruits and vegetables will give us useful nutritional information as well as their possible antioxidant mechanism(s).

Recently, several methods, which were based on on-line detection of antioxidants by post-column neutralization of eluates with free radicals, have been reported to be successfully utilized to identify and quantify antioxidants in certain biological samples [11–15]. However, the limitations of these methods are obvious when standards of unknown antioxidant components are not available and the reaction stoichiometry of an antioxidant and free radical is still not clear. Therefore, it is difficult to measure and identify the major antioxidant(s) in biological samples using these existing methods. We had further successfully characterized and identified antioxidant(s) in star fruit by comparing chromatogram of sample extract with oxidized sample extract by $ABTS^{\bullet+}$, which is prepared with ABTS and potassium persulfate [16]. However, a few background peaks were found in the chromatograms. These background peaks might interfere with the characterization of some antioxidant peaks. In this paper, we are reporting an improved approach using $ABTS^{\bullet+}$, prepared by oxidation of ABTS with MnO_2, to characterize antioxidant peaks, and thus reduce interference peaks obtained from $ABTS^{\bullet+}$ solution in the spiking tests. The improved approach was systematically used to analyze major antioxidants in lady's finger, a common vegetable in Southeast Asia. Lady's finger is a native of tropical Africa and is now widely grown in the tropics and the sub-tropics. It has been reported that lady's finger is a good source of trace elements such as zinc, copper, magnesium, calcium and selenium [17]. Surprisingly, its antioxidant capacity and major antioxidant components are not reported yet. This study covers characterization of major antioxidants in lady's finger, structure elucidation of characterized antioxidants using tandem mass spectrometry and their structure confirmation using nuclear magnetic resonance spectroscopy.

2. Experimental

2.1. Reagents and materials

2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), manganese dioxide, sodium methoxide, aluminum chloride, boric acid, sodium carbonate anhydrous, quercetin, (−)-epigallocatechin were purchased from Sigma (St. Louis, MO, USA); methanol and hydrochloric acid from Merck (Darmstadt, Germany).

Fresh lady's finger was purchased from Pasir Panjang Wholesale center.

2.2. Preparation of sample extracts

Fresh lady's finger was homogenized and immediately extracted in 70% denatured ethanol (w/v, 1:8) for 4 h with continuous stirring in a dark bottle. The extract was filtered. The filtrate was kept in the fridge at 4 °C. The residue was extracted again using one fourth of the first extraction volume. The two filtrates were combined and solvent was evaporated under vacuum at 35 °C, and then redissolved in pure water. It was found that the loss of TAA was less than 5% after evaporation and reconstitution. Hexane was subsequently added to remove non-polar compounds, e.g. carotenoids and chlorophyll. There was almost no loss of antioxidant during hexane extraction process. The aqueous portion was collected and dried by vacuum evaporation at 35 °C. Forty per cent of aqueous methanol was used to top up the volume. The solution was stored in the fridge at 4 °C for 2 days to further remove chlorophyll precipitated from the solution. The obtained solution was centrifuged at 4 °C and filtered with 0.45 μm membrane filter and then used for TAA, HPLC, HPLC–MS and semi-preparative HPLC analyses.

2.3. Solid-phase extraction of antioxidants

The extract was subjected to solid-phase extraction using a reversed-phase C_{18} column (Isolute) that has been conditioned with pure water. One milliliter of juice was introduced to column. Half milliliters of water was used to elute most of the more polar components (FRC 1), and then 1 mL of methanol was used to elute and the fraction was collected (FRC 2), and then another 1 mL of methanol was used to elute possibly-existing more non-polar compounds (FRC 3). The TAA of all the fractions collected and extracts were measured by $ABTS^{\bullet+}$ scavenging assay. The fractions were also used for HPLC assay.

2.4. $ABTS^{\bullet+}$ scavenging assay

The TAA assay was carried out on the Ultraspec 3000 UV–vis spectrophotometer (Pharmacia Biotech, Cambridge, UK). $ABTS^{\bullet+}$ solution was prepared as previously reported by Liaga and Lissi [18]. The procedure for TAA assay was as described by Leong and Shui [19]. The results were expressed as mg/100 g L-ascorbic acid equivalent antioxidant capacity (AEAC).

2.5. HPLC characterization of major antioxidant peaks

Chromatographic separations were done on a Shim-Pak VP-ODS column (250 mm × 4.6 mm i.d.) (Shimadzu, Kyoto, Japan) with a diode array detection (DAD) system under

the following elution conditions: flow rate = 600 μL min^{-1}; 30 °C; solvent A, 0.1% formic acid in water; solvent B, methanol; starting from 30 to 70% B in 30 min and keeping constant for 4 min, and then from 70 to 30% B in 6 min and keeping constant for 5 min for reconditioning the column.

One milliliter of extract and 5 mL of $ABTS^{\bullet+}$ solution are mixed to reacted for 1 h and then passed through 0.45 μm filter and used for HPLC assay. Blanks of juice with water and $ABTS^{\bullet+}$ with water were also analysed.

In addition, 20 (L of extract from fresh lady's finger was injected into the HPLC to obtain L-ascorbic acid (AA) contribution using a previously-developed method [20].

2.6. HPLC–DAD–ESI-MS^n analysis of antioxidants

The apparatus was a Finnigan/MAT LCQ ion trap mass spectrometer (San Jose, CA, USA) equipped with TSP spectra system, which includes a UV6000LP DAD system, P4000 quaternary pump and AS3000 autosampler. The heated capillary and voltage were maintained at 200 °C and 4.5 kV, respectively. The full scan mass spectra from *m/z* 50–2000 were recorded. Nitrogen is operated at 80 psi for sheath gas flow rate and 20 psi for auxiliary gas flow rate (1 psi = 6894.76 Pa). The full scan mass spectra from *m/z* 50–2000 were acquired both in positive and negative model with a scan speed of 1 s/scan. Tandem mass spectrometry was performed using helium as collision gas, operated at 0.8 mTorr, and the collision energy was set from 10 to 100% and optimized collision energy was chose for individual compounds (1 Torr = 133.322 Pa).

For HPLC–DAD–ESI-MS assay of extract, the instrument was set to measure the following events: (1) UV spectra of individual peaks; (2) TICs; (3) zoom scan was applied for measuring isotopic distances at *m/z* 597, 627, 465, and 551, respectively; (4) MS–MS and MS^n were applied to break down the most abundant $[M+H]^+$ or $[M-H]^-$ and daughter ions from MS^{n-1}. Chromatographic separations were done on a Shim-Pak VP-ODS column (250 mm × 4.6 mm i.d.) (Shimadzu, Kyoto, Japan) with a guard column (GVP-ODS, 10 mm × 4.6 mm i.d.) under the following elution conditions: flow rate, 600 μL min^{-1}; room temperature (27 °C); solvent A, 0.1% formic acid in water; solvent B, methanol, starting from 20 to 50% B in 20 min, from 50 to 90% B in 25 min, and from 90 to 20% B in 5 min and keeping constant for another 5 min for washing and reconditioning of the column.

2.7. Isolation of pure compounds by semi-preparative HPLC

Two milliliters of sample solution was loaded on a tandem column system, which consists of a C_{18} Nova-Pack C_{18} (300 mm × 7.8 mm, Waters) and Shim-Pak VP-ODS column (250 mm × 4.6 mm i.d.) (Shimadzu, Kyoto, Japan). Isocratic flow rate 1.2 ml/min, 40% of methanol and 60% of 0.1% formic acid were the eluents. Compounds **1**–**4** were isolated from the extract by means of repeated semi-preparative HPLC. The purity of the compounds was examined by analytical HPLC–DAD.

2.8. Spectroscopy study of isolated compounds

General UV–vis shift reagents were prepared according to Markham for UV–vis absorption spectroscopy [21]. NMR spectra were acquired using a Bruker DPX300 spectrometer and Bruker AMX500 spectrometer. Samples were dissolved in [$^2H_6$] dimethyl sulfoxide (DMSO-d6), and reference to the residual solvent resonance at δ_C = 39.50 ppm as appropriate.

3. Results and discussions

3.1. Characterisation of major antioxidants in aqueous ethanol extract of Lady's finger by HPLC–DAD

3.1.1. Characterisation of antioxidant peaks by spiking with free radicals

As shown previously, several background peaks were eluted from $ABTS^{\bullet+}$ solution, which was prepared by oxidation of ABTS by potassium persulfate [16]. Here, it was found that no peaks were eluted from $ABTS^{\bullet+}$ solution prepared by oxidizing ABTS with MnO_2. Thus, $ABTS^{\bullet+}$ solution prepared by reaction with MnO_2 is a better choice for characterizing antioxidant peaks as no interference is introduced from $ABTS^{\bullet+}$ solution throughout the period monitored (Fig. 1). Peak heights of antioxidant peaks were significantly reduced after reaction with $ABTS^{\bullet+}$ (Fig. 2). As shown in Fig. 2, several major peaks, which had retention times between 20 and 30 min, were initially identified as major antioxidant components as those peaks disappeared or were significantly reduced after reacting with

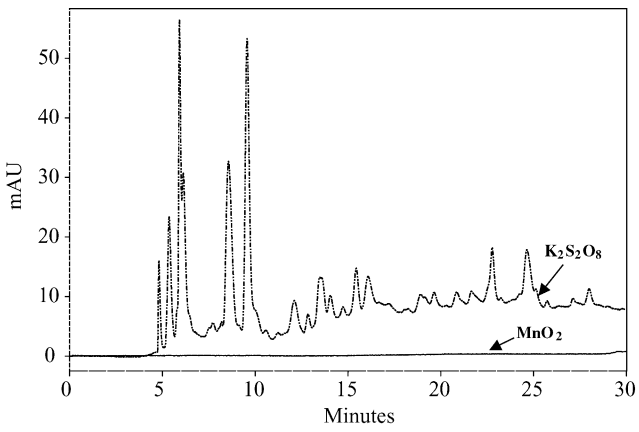

Fig. 1. Chromatograms of $ABTS^{\bullet+}$ solution oxidized by potassium persulfate and manganese dioxide. Solid line, $ABTS^{\bullet+}$ oxidized by manganese dioxide; dashed line, $ABTS^{\bullet+}$ oxidized by potassium persulfate.

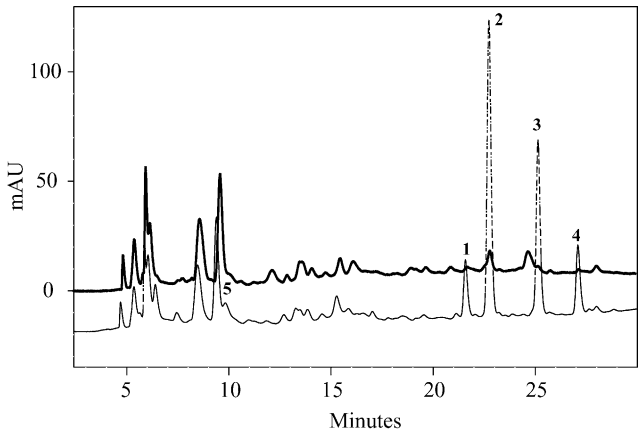

Fig. 2. Chromatograms of extract and reaction solution of extract and ABTS$^{\bullet+}$. Solid line, 1:5 of extract/ ABTS$^{\bullet+}$ (v/v); dashed line, 1:5 of extract/0.1% formic acid (v/v). Detection wavelength: 280 nm.

free radicals. The four antioxidants, which had retention times between 20 and 30 min, were initially identified as major antioxidants and named as compounds **1**–**4** according to their elution order. In addition, one small peak, which was eluted at around 9.5 min and named compound **5**, was also identified as an antioxidant since it was found to react with ABTS$^{\bullet+}$.

3.1.2. Elution period of major antioxidant peaks by solid phase extraction (SPE)

Table 1 lists the antioxidant activities of FRC 1 to FRC 3 and their percentage contributions to TAA. As shown in the table, FRC 2 accounted for around 70% of the antioxidant activities of all the fractions.

Chromatograms of FRC 1, FRC 2 and FRC 3 showed that FRC 2 mainly collected those peaks with retention times from 20 to 30 min. Other non-polar compounds, which may exist in the extract, do not show any antioxidant activity (Fig. 3). Therefore, the major antioxidant peaks were the four major peaks, compounds **1**–**4**, as shown in Fig. 3. In this study, we do not observe any other antioxidant peaks when examined within the UV–vis range when the extracts were spiked with ABTS$^{\bullet+}$. In addition, TAA assay of pure compounds isolated from one batch of lady's finger using semi-preparative HPLC shows that sum of antioxidant activity of four quercetin derivatives is close to 70% of TAA of the plant extract (data not shown). This strongly supports that those characterized peaks are major antioxidant peaks in plant extract.

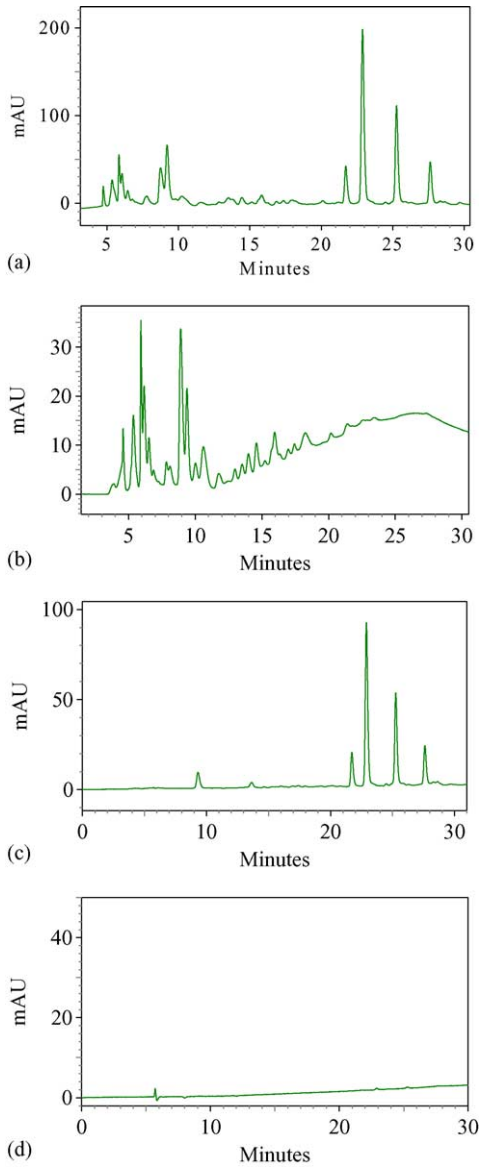

Fig. 3. Chromatograms of extract and FRCs. (a) 1:3 of extract/0.1% formic acid; (b) FRC 1; (c) 1:1 of FRC 2/0.1% formic acid; (d) FRC 3. Detection wavelength: 280 nm.

3.2. Identification of antioxidant components using HPLC coupled with tandem mass spectrometry

Results from HPLC–DAD shows that the major antioxidant components **1**–**4** all have similar spectra to that of

Table 1
Antioxidant activities of solvent extracted fractions

	FRC 1	FRC 2	FRC 3	Original extract
Vol. (mL)	1.20±0.07	0.91±0.08	0.98±0.04	1.0
ΔA_{414nm}	0.217±0.041	0.697±0.055	0.009±0.001	0.816±0.003
AC (vol. × $\Delta A_{414\,nm}$)	0.260	0.634	0.009	0.816
TAA%	31.9 (28.8[a])	77.7 (70.2[a])	1.1 (1.0[a])	

[a] The percentage contribution to TAA in bracket was modified according to that the sum of TAA% by FRCs is 100%.

quercetin (R_t = 29 min). In addition, acidic hydrolysis releases quercetin aglycones. Thus, they were taken as quercetin derivatives. The procedure for structure elucidation of those compounds may involve (1) confirmation of the existence of the quercetin aglycone; (2) identification of the substitution group and its substitution position on the aglycone.

As discussed above, FRC 2, which accounted for 70 % of TAA, mainly collected compounds **1**–**4**. Although HPLC–DAD provided good separation of compounds interested and give much information on the possible class of these compounds, it is not able to confirm the compound without authentic standards. As reviewed previously, mass spectrometry and HPLC coupled with mass spectrometry are very powerful tools for identification and structural determination of naturally occurring substances including flavonoids and related compounds [22]. The obvious advantage of HPLC–MS is that it provides the possibilities of identifying different compounds in a complex sample. The structural elucidation of compounds **1**–**4** and another antioxidant compound **5** using HPLC–MS will be discussed below.

Compounds **1**–**4** as well as **5** are the major antioxidant components in lady's finger. In HPLC–MS, the same chromatographic conditions as those applied in HPLC–DAD assay were used, total ion chromatogram of the plant extract was similar to that of plant extract in Fig. 2. All peaks of interest are well separated and further characterized by tandem mass spectrometry. As HPLC–MS is only used for structure elucidation of antioxidant compounds and not for reaction product, the reaction mixture is not used for HPLC–MS assay. Compound **5** was identified as (−)-epigallocatechin by spiking sample with real standard and comparing its mass spectra and CID spectra with those of authentic standard (Table 2).

Table 2 also gives the HPLC–DAD–ESI-MS^n (n = 1–4) mass spectra of compounds **1**–**4**. Other than major ion peaks $[M + H]^+$ in HPLC–MS mode, other ion peaks such as $[M + Na]^+$, $[M + M + H]^+$, $[M + M + Na]^+$ and ion at m/z 303 were also observed. The ESI mass spectra of compounds **1**–**4** all includes common peak ion at m/z 303, which is corresponding to the ion peak of quercetin aglycone. The structural information can be enhanced by using tandem MS with collision-induced dissociation (CID). Compounds **1**–**4** gave similar CID spectra for ion peak m/z 303 as shown in Table 2. Further CID spectra at m/z 303 were compared to the MS–MS results for the quercetin. The results confirmed that they were different derivatives of quercetin. Therefore, the aglycone of compound **1**–**4** was identified as quercetin. Their structural information could be further obtained using HPLC–ESI–MS^n and other techniques.

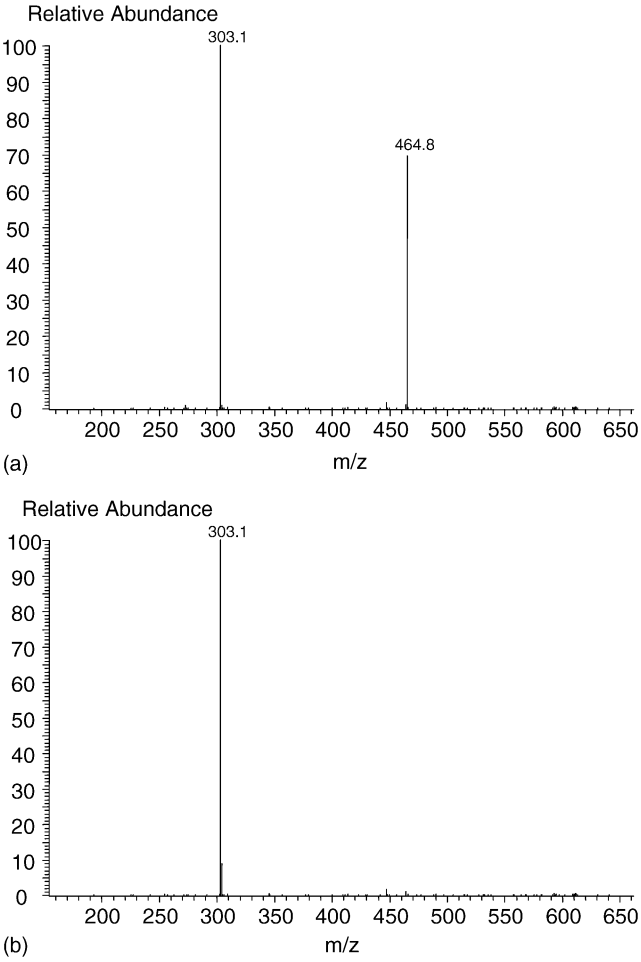

Fig. 4. ESI-MS^2 mass spectra of compounds at positive mode. (a) CID spectrum corresponds to the positive parent ion(s) at m/z 597 (MS/MS Scan), collision energy: 50%; (b) CID spectrum corresponds to the positive parent ion(s) at m/z 551 (MS/MS Scan), collision energy: 50%. CID spectra at m/z 627 and 465 are similar to those at m/z 597 and 551, respectively.

Fig. 4 shows the MS/MS spectra of parent ions at $[M + H]^+$. For compound **1**, the daughter ions at m/z 465 and

Table 2
ESI-MS^n spectra of compounds **1**–**5**

Compounds	ESI-MS[a]	ESI-MS^{2}[b]	ESI-MS^{3}[b]	ESI-MS^{4}[b]
1	*597* (+)	*465*, 303	*303*	257, 285, 229, etc.
2	*627* (+)	*465*, 303	*303*	257, 285, 229, etc.
3	*465* (+)	*303*	257, 285, 229, etc.	
4	*549* (−)	*505*	*463*, 301, 300	301, 300
	551 (+)	*303*	257, 229, 285, etc.	
5	*307* (+)	139, 151, 169, 181, 289		

[a] Only $[M + H]^+$ (100%) were listed, other ion peaks were $[M + Na]^+$, $[M + M + H]^+$, $[M + M + Na]^+$.
[b] CID spectra were obtained with the previously italicised bold ions.

303 by ESI-MS^2 and ESI-MS^3, respectively, indicated sequential loss of pentose and hexose (Table 2). In addition, high-resolution electron ionization MS (HR-EI-MS) gave the molecular formula of compound **1** to be $C_{26}H_{28}O_{16}$. Therefore, the most possible structure of compound **1** is quercetin–hexose–pentose.

For compounds **2**–**4**, HR-EI-MS gave their molecular formulae to be $C_{27}H_{30}O_{17}$, $C_{21}H_{20}O_{12}$ and $C_{24}H_{22}O_{15}$, respectively. According to the CID spectra of their parent ions, loss of hexose in compound **3** and sequential loss of hexose in compound **2** indicated compounds **2** and **3** to be quercetin–hexose–hexose and quercetin–hexose, respectively. For compound **4**, its CID spectra from parent ions at *m*/*z* 551 (positive), which were observed at different collision energy levels, only gave the information of the existence of quercetin aglycone. However, its CID spectra from parent ions at *m*/*z* 549 (negative) and 505 (daughter ion of parent ions at *m*/*z* 549) indicated sequential loss of carboxyl group (CO_2), C_2H_2O group and hexose group (Fig. 5). These results indicated that the main fragmentation pattern in negative mode was related to cleavage of the acylation bond between hexose and malonic acid. Therefore, compound **4** was tentatively identified as malonylated–hexose–quercetin.

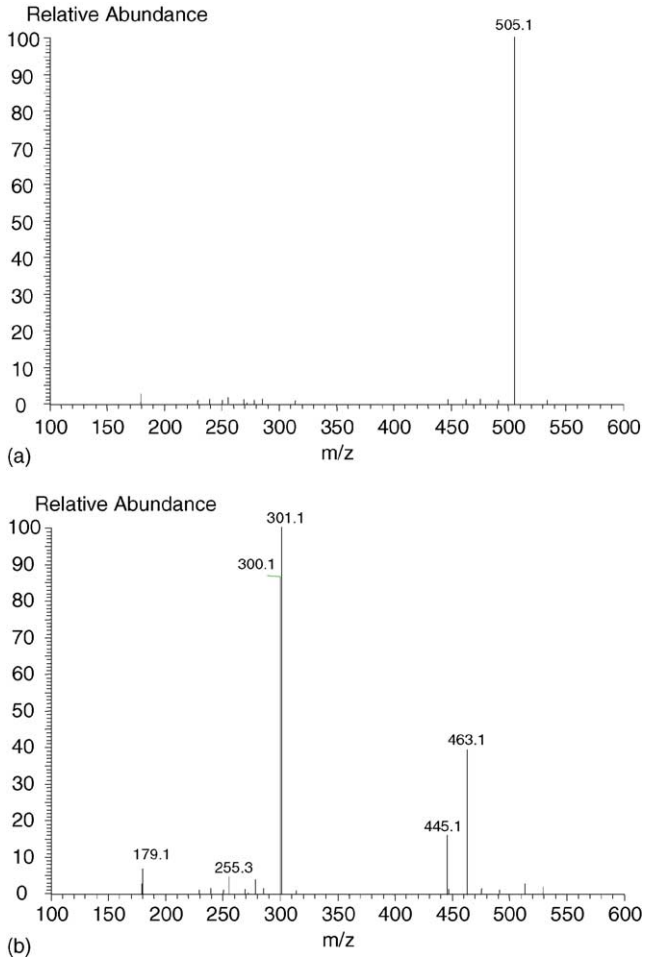

Fig. 5. ESI-MS^n mass spectra of compounds **4** at negative mode. (a) CID spectrum corresponds to the negative parent ion(s) at *m*/*z* 549 (MS/MS Scan), collision energy: 80%. (b) CID spectrum corresponds to the negative parent ion(s) at *m*/*z* 505 from *m*/*z* 549 (MS^3), collision energy: 80%.

3.3. Structure confirmation using spectrometric methods

As discussed above, (−)-epigallocatechin and the molecular formulae of four quercetin derivatives were identified using HPLC combined with ESI-MS^n techniques. However, it is still not clear about the glycosylation position between sugar and aglycon and acylation position between sugar and acid.

Compound **1**: UV λ_{max}^{MeOH}: 357, 256; +NaOMe 404, 325, 273; +$AlCl_3$433, 273; +$AlCl_3$ + HCl 398, 269; +NaOAc 382, 270; +NaOAc + $H_3BO_3$378, 262 nm. The UV–vis spectra of compounds **2**, **3** and **4** in MeOH also showed absorption maxima at around 256 and 357 nm with similar shifts after addition of various reagents. The UV spectral changes induced by the various shift reagents indicated that the flavonoid had no free hydroxyl group at C-3 and free hydroxyl groups at C-5, C-7, C-3′ and C-4′ [21]. In addition, their ^{13}C NMR spectroscopic data in DMSO-d_6 also confirmed that their aglycone is quercetin and glycosylation occurs at C-3 position. Table 3 listed the ^{13}C NMR spectral data for compound **1** (300 MHz, DMSO-d_6), **2** (500 MHz, DMSO-d_6), **3** (300 MHz, DMSO-d_6) and **4** (300 MHz, DMSO-d_6).

As discussed above, compound **1** was quercetin monodesmoside with one inner hexose moiety bonded at C-3 and one terminal pentose moiety. The NMR spectra of compound **1** (Table 3) were resolved by "best fit" matching to appropriate monosaccharide spectra or through a comparative study of the simpler mono- or di-glucosides that consistute part of the structure. The assignments in the spectrum of compound **1** were deduced by reference to the ^{13}C NMR spectra of a number of flavonol monodesmosides [23], luteolin 3-xylosyl (1‴ → 2″) glucoside [24], quercetin 3-*O*-glucoside (3) and kampferol 3-*O*-xylosyl (1‴ → 2″) glucoside [25]. Glucose was assigned to be the inner sugar while the terminal sugar was identified as xylose. The other possible combination of hexose and pentose will give much more differences on 13C NMR spectra [23]. Compared with the NMR spectra of quercetin 3-*O*-glucoside (3), a downfield shift of C-2" (glc) at 81.9 ppm was observed due to 1 → 2 intersugar linkage. The assignments of xylosyl carbons are almost identical to those of the counterparts of apigenin 7-*O*- xylosyl (1‴ → 2″) glucoside and luteolin 3-xylosyl (1‴ → 2″) glucoside [24]. Therefore, compound **1** was assigned to be quercetin 3-*O*-xylosyl (1‴ → 2″) glucoside. Other than carbons from quercetin aglycone, the ^{13}C NMR spectra data of compound **2** (Table 3) also revealed the presence of two sugar molecules with a total of 12 carbon signals. The chemical shifts of 12 sugar carbons were almost identical to those of the counterparts of two flavonol 3-*O*-glucosyl (1‴ → 6″) glucoside [23,26]. A downfield shift of C-6″ at 68.0 was due to a 1 → 6 linkage between two sugars. The compound **3** was identified as quercetin–hexose by ESI-MS^n. Its ^{13}C NMR spectra

Table 3
^{13}C NMR spectral data of compounds **1**–**4** in DMSO-d_6 (δ: ppm)

Carbon number	1	2	3	4
Flavonoid moiety				
C-2	155.2	156.2	156	156.4
C-3	132.9	133.2	133.2	133.2
C-4	177.3	177.2	177.3	177.4
C-5	161.2	161.1	161.2	161.2
C-6	98.7	98.8	98.7	98.8
C-7	164.2	164.6	164.6	164.4
C-8	93.4	93.6	93.5	93.7
C-9	156.2	156.4	156.3	156.7
C-10	103.7	103.8	103.7	103.9
C-1′	121.0	121.0	121.1	121.1
C-2′	115.2	115.2	115.2	115.2
C-3′	144.9	144.8	144.8	145.0
C-4′	148.6	148.5	148.5	148.6
C-5′	115.9	116.1	116.1	116.3
C-6′	121.8	121.6	121.5	121.5
Sugar moiety				
C-1″	97.9	100.8	100.9	101.1
C-2″	81.9	73.3	74.05	74.0
C-3″	76.9	76.5	76.5	76.8
C-4″	69.6	69.6	69.9	69.6
C-5″	77.6	76.3	77.5	76.8
C-6″	61.3	68.0	60.9	69.6
C-1‴	104.6	103.1		
C-2‴	73.9	74.0		
C-3‴	76.1	76.3		
C-4‴	69.4	69.7		
C-5‴	65.7	76.4		
C-6‴		60.7		
Malonyl moiety				
C-7″				166.9
C-8″				41.8
C-9″				167.9

Compound **1**: quercetin 3-*O*-xylosyl (1‴ → 2″) glucoside; compound **2**: quercetin 3-*O*-glucosyl (1‴ → 6″) glucoside; compound **3**: quercetin 3-*O*-glucoside; compound **4**: quercetin 3-*O*-(6″-*O*-malonyl)-β-glucoside.

(Table 3) in DMSO-d_6 were consistent with those of quercetin 3-*O*-glucoside [27]. Similarly, the ^{13}C NMR spectroscopic data of compound **4** (Table 3) was consistent with quercetin-3-*O*-(6-malonylglucoside) [28,29].

Chemical structures of compounds **1**–**4** are shown in Fig. 6.

3.4. Some precautions of using this approach

Antioxidants, which usually work as effective free radical scavengers, most likely have UV–vis absorption due to their unsaturated bond(s) and thus most likely could be monitored by a DAD system. A DAD system is able to record chromatograms at any wavelength(s) within UV–vis range, and thus could minimize the chances of some compounds missing from detection due to poor molar extinction coefficients at a specific wavelength. A post-run check of chromatograms of plant extract and reaction mixture at different wavelengths is necessary for characterization of different variety of antioxidants. While this improved approach was successfully applied to identify major antioxidants in aqueous ethanol extract of lady's finger, care should be taken when sum of total antioxidant capacity of SPE fractions were much lower than original plant extract. In such a situation, conditions of solid phase extraction, such as cartridge and solvent, should be improved to recover as much as possible antioxidants from the cartridge. In addition, in some cases, it may be observed that intensity of antioxidant peaks does not tally total antioxidant capacity. This might arise from the fact that some antioxidants may not be detected during HPLC analysis under selected experimental conditions, such as mobile phase, column and detection system. In that case, improved HPLC conditions might help resolve this problem.

While this study studied major antioxidants of aqueous ethanol extract of lady's finger, the plant might also contain other variety of antioxidants, which might not be extractable

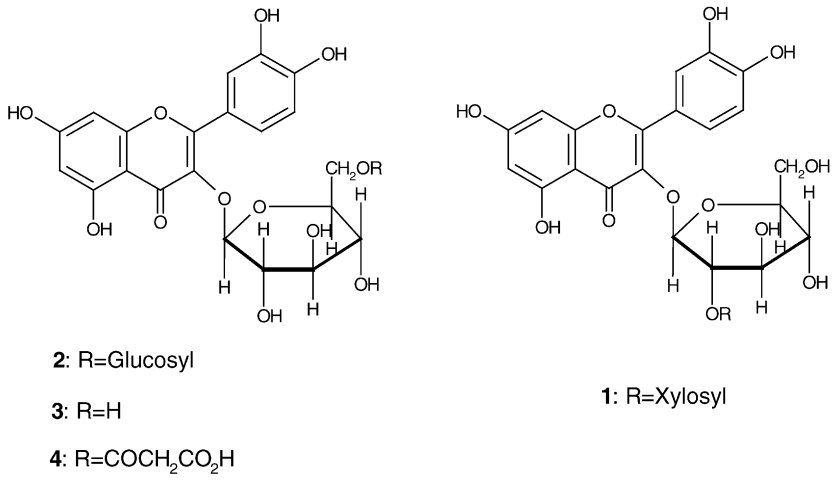

Fig. 6. Chemical structures of compounds **1**–**4**.

here, such as carotenoids, etc. It should be noted that TAA of the plant might be much higher than that contained in aqueous ethanol extract.

4. Conclusions

The improved approach, which mainly involved (1) identification of major antioxidants using HPLC analysis of biological samples, $ABTS^{\bullet+}$ solution prepared by oxidizing ABTS with MnO_2, reaction solution of both and fractions from SPE and TAA assay of related solution and (2) structural elucidation of major antioxidants using modern techniques, was successfully applied for identification of major antioxidants in lady's finger. In addition, this approach has also been successfully applied on a few other fruits and vegetables (papers in progress). We believe that this approach can be used for identification of major antioxidants in a variety of biological samples.

To our knowledge, the four quercetin derivatives and (−)-epigallocatechin were first identified in lady's finger. As flavonoids have attracted increasing attention due to their potential health benefits, the next step of our research is to quantify them for the initial understanding of the possible dietary intake of these compounds. Further research on stoichiometry and kinetic study of isolated compounds are still on going.

Acknowledgements

The authors are grateful to A/P Philip J. Barlow for his suggestions, Madam Frances Lim for kindly providing preparative HPLC instrument and column, Madam Wong Lai Kwai for HR-EI-MS data acquisition, Madam Han Yanhui and Ms Ler Peggy for 500 MHz NMR data acquisition.

References

[1] B. Halliwell, J.M.C. Gutteridge, Free Radicals in Biology and Medicine, Oxford University Press, Oxford, 1999, pp. 10–11.
[2] A.T. Diplock,Molecular Aspects of Medicine, vol. 15, Pergamon Press, Oxford, New York, 1994, pp. 295–376.
[3] H. Wiseman, B. Halliwell, Biochem. J. 313 (1996) 17.
[4] B.N. Ames, Dietary 221 (1983) 1256.
[5] B.N. Ames, M.K. Shigenaga, T.M. Hagwn, Proc. Natl. Acad. Sci. (U.S.A.) 90 (1993) 7915.
[6] B. Halliwell, Ann. Rev. Nutr. 16 (1996) 33.
[7] A.D. Haegele, C. Gillette, C. O'Neill, P. Wolfe, J. Heimendinger, S. Sedlacek, H.J. Thompson, Cancer Epidemiol. Biomarkers Prevent. 9 (2000) 421.
[8] D. Feskanich, R.G. Ziegler, D.S. Michaud, E.L. Giovannucci, F.E. Speizer, W.C. Willett, G.A. Colditz, J. Natl. Cancer I 92 (2000) 1812.
[9] K.B. Michels, E. Giovannucci, K.J. Joshipura, B.A. Rosner, M.J. Stampfer, C.S. Fuchs, G.A. Colditz, F.E. Speizer, W.C. Willett, J. Natl. Cancer I 92 (2000) 1740.
[10] R.L. Prior, G. Cao, Free Radical Biol. Med. 27 (1999) 1173.
[11] I.I. Koleva, H.A.G. Niederlander, T.A. van Beek, Anal. Chem. 72 (2000) 2323.
[12] I.I. Koleva, H.A.G. Niederlander, T.A. van Beek, Anal. Chem. 73 (2000) 3373.
[13] A. Dapkevicius, T.A. van Beek, H.A.G. Niederlander, J. Chromatogr. A 912 (2001) 73.
[14] A. Dapkevicius, T.A. van Beek, H.A.G. Niederlander, Anal. Chem. 71 (2000) 736.
[15] D. Bandoniene, M. Murkovic, J. Age. Food. Chem. 50 (2002) 2482.
[16] G. Shui, L.P. Leong, J. Chromatogr. A 1022 (2004) 67.
[17] T.S. Srikumar, Food Chem. 46 (1993) 163.
[18] C. Liaga, E.A. Lissi, Can. J. Chem. 78 (2002) 1052.
[19] L.P. Leong, G. Shui, Food Chem. 76 (2002) 69.
[20] G. Shui, L.P. Leong, J. Chromatogr. A 977 (2002) 89.
[21] K.R. Markham, Techniques of Flavonoid Identification, Academic Press, London, 1982.
[22] M. Careri, F. Bianchi, C. Corradini, J. Chromatogr. A 970 (2002) 3.
[23] P.K. Agrawal, Carbon-13 NMR of Flavonoids, Elsevier, New York, 1989.
[24] M. Parveen, M.S. Khan, Shafiullah, M. Ilyas, Phytochemistry 49 (1998) 2535.
[25] N. Tanaka, H. Yuhara, H. Wada, T. Murakami, R.C. Cambie, J.E. Braggins, Phytochemistry 32 (1993) 1037.
[26] A. Masakazu, K. Tetsuya, K. Toshio, Phytochemistry 25 (1985) 231.
[27] J.H. Lin, Y.T. Lin, J. Food Drug Anal. 7 (1999) 185.
[28] K. Azuma, M. Nakayama, M. Koshioka, K. Ippoushi, Y. Yamaguchi, K. Kohata, IH. Higashio, J. Agric. Food Chem. 47 (1999) 3963.
[29] B. Wald, R. Wray, K. Galensa, K. Herrmann, Phytochemistry 28 (1989) 663.