the high boiling temperature of diuron, a pre-concentration factor of 1000 was gained with the liquid–liquid extraction technique as described in the Experimental section. Preliminary experiments in triplicate, performed for standard solutions prepared by addition of 0.01 μg in 1000 ml of ultrapure water, gave a recovery of 87 ± 2%. The limit of determination was determined by standard addition method and was found to be 10 μg/l.

Fig. 4A shows the chromatogram obtained for the 1000-fold preconcentrated sample of lagoon water. It can be seen that also in the concentrated sample the time window corresponding to the herbicide retention is interference free. By the standard addition method (an example is

shown in Fig. 4B for the addition of 50 μg/l) a concentration of diuron of 42 ± 6 μg/l was estimated in the concentrated sample. This corresponds to an amount of at least 42 ng/l in the native sample.

As a conclusion, the method developed for the separation and determination of phenylurea herbicides is very suitable–also without preconcentration steps–for the analysis of surface waters with detection limits lower than 9 μg/l. The results obtained for a lagoon water sample show that with a preconcentration step, the method could be advantageously employed in the analysis of drinking water, taking also into account its much lower matrix interference with respect to sea water.
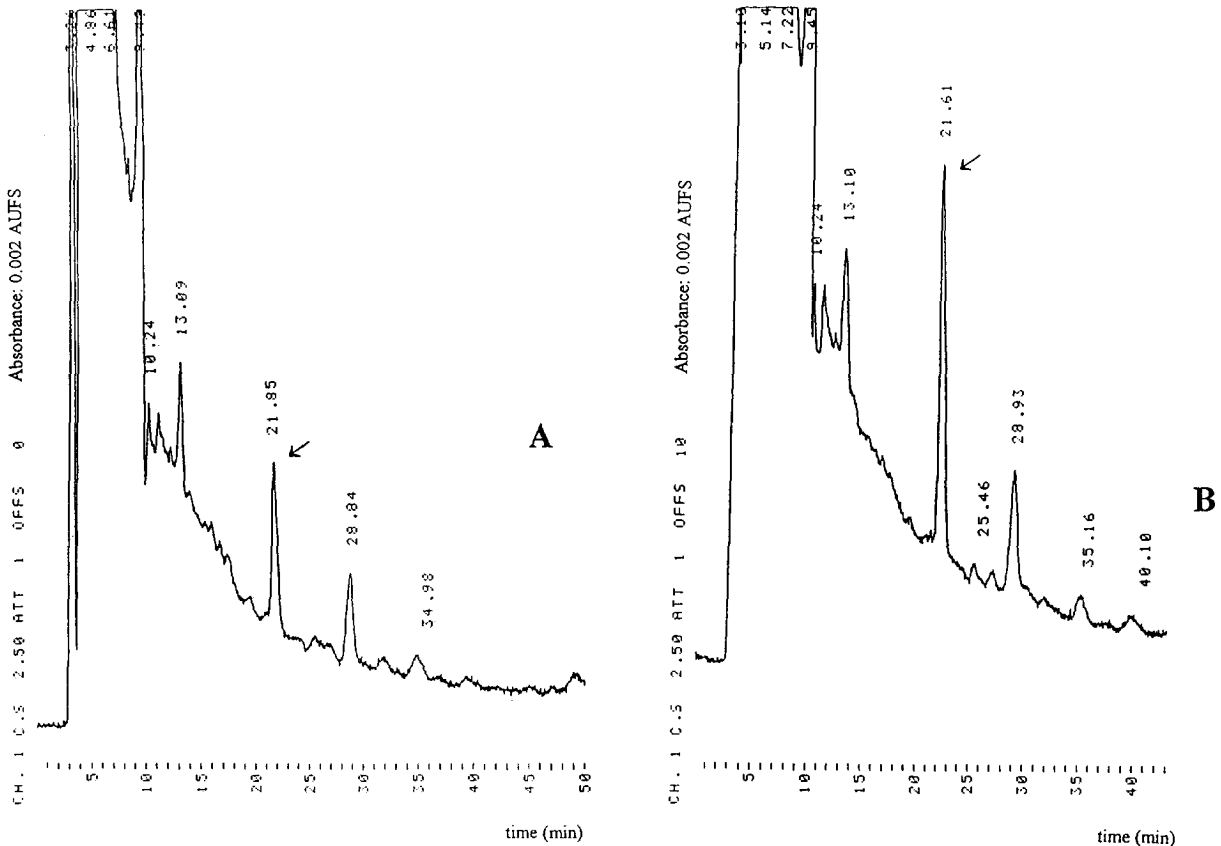

Fig. 4. Chromatographic analysis of the lagoon water sample after 1000-fold preconcentration. Experimental conditions as in Fig. 3. The arrows indicate the diuron peak before (A) and after (B) spiking with 50.0 μg/l of diuron.

Acknowledgement

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Identification of nonvolatile components in lemon peel by high-performance liquid chromatography with confirmation by mass spectrometry and diode-array detection

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Abstract

High-performance liquid chromatography (HPLC) with diode-array detection and thermospray mass spectrometry (LC–TSP-MS) were used to identify nonvolatile compounds in a lemon peel extract. In this way, the retention time characteristics, the UV–Vis spectra and the mass spectra provide structural information without the necessity of isolating the individual compounds. The lemon peel extract was separated into petroleum ether, chloroform, ethyl acetate and butanol fractions. The chloroform fraction was found to contain mainly the limonoids; the petroleum ether fraction contained coumarins, phenyl propanoid glycosides and flavones-C-glucosides; and the ethyl acetate fraction which had the best antioxidant activity, was found to contain flavonols, flavones-O-glycosides and flavanones.

1. Introduction

The chemical components of *Citrus* have an important role in the human diet and in human health. Their effectiveness in decreasing erythrocyte aggregation and blood coagulation in vitro [1] and, for some of them, the anticarcinogenic activity in vitro and in vivo are well known [2]. In the genus *Citrus*, the lemon (*Citrus limon* Burm. *f.*) is one of the most important crops.

The flavonoid content of lemon peel has been studied since 1936 when Szent-Gyorgy [3] extracted "citrin", a mixture of lemon flavonoids formerly regarded as a vitamin. Since then, many studies have been conducted on this subject. The lemon peel bioflavonoids are characterized by the presence of four groups of flavonoids: flavones-O-glycosides, flavones-C-glycosides, flavonols, flavanones. The first group is very common among fruits, and in the lemon peel luteolin-7-rutinoside and diosmetin-7-rutinoside (diosmin) have been found [4].

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Flavones-C-glycosides are broadly distributed in the plant kingdom [5]. Park et al. [6] found four flavones-di-C-glycosides in the lemon peel: 6,8-di-C-glucosyl-luteolin; 6,8-di-C-glucosyl-apigenin; 6,8-di-C-glycosyl-chrisoeriol; 6,8-di-C-glucosyl-diosmetin. The flavonol group in lemon peel is characterized by the presence of rutin (quercetin-3-O-rutinoside) and three poly-methoxylated compounds: 3,5,7,4'-tetrahydroxy-6,8,3'-trimethoxy-flavone (limocitrol); 3,5,7,4'-tetrahydroxy-8,3'-dimethoxy-flavone (limocitrin); 3,5,7,3'-tetrahydroxy-6,8,4'-trimethoxy-flavone (isolimocitrol). These are less polar compounds compared to the other lemon bioflavonoids. Polymethoxylated flavones are present especially in the genus *Citrus* and are located in the flavedo [7]. These latter compounds are extremely interesting because of their physiological effects on animal and man. They have effect on high blood viscosity syndrome and, in addition, they possess antimicrobial and antiviral activity [8]. The last group is that of the flavanones. These compounds are very important in the genus *Citrus* because of their chemotaxonomic properties [9] and also because of their relation to taste and bitterness. The compounds present in lemon peel are the following three glycosides: hesperitin-7-rutinoside (hesperidin), naringenin-7-rutinoside and eriodictyol-7-rutinoside (eriocitrin) [10,11]. Several other groups of polyphenols are known to be present in lemon peel. Among those, phenolic acids and phenyl propanoids (coumarins, phenyl propanoid glycosides) have been reported [7]. The objective of this study was to develop a fast and reliable method to determine the presence and distribution of the various components in a lemon peel extract. High-performance liquid chromatography (HPLC) with diode-array detection (DAD) and thermospray mass spectrometry (LC–TSP-MS) were used to identify several compounds. In this way, the retention time characteristics, the UV–Vis spectra and the mass spectra provide structural information without the necessity of isolating the individual compounds. The methodology included a series of liquid–liquid extractions and optimization of the chromatographic conditions for HPLC–DAD and HPLC–MS analysis for each extract. Finally, the antioxidant activity of each extract was evaluated.

2. Experimental

2.1. Sample preparation

A dried lemon peel bioflavonoid extract (M. Phil Yen, New York, NY, USA) was used as raw material to analyze the flavonoid content. An amount of 2 g of lemon peel was extracted three times with 50 ml of 80% ethanol containing 0.1% HCl. The extracts were filtered with Whatman No. 1 filter paper and the ethanol was removed with a rotary evaporator at 30°C under vacuum. The residue was dissolved in 50 ml of water and extracted three times with 50 ml of petroleum ether, chloroform, ethyl acetate and water-saturated butanol, respectively (see Fig. 1). Solvents from all fractions were removed with a rotary evaporator at 30°C under vacuum to obtain the petroleum ether extract, chloroform extract, ethyl acetate extract and butanol extract. The residues were dissolved in 2 ml of water–methanol (1:1) for HPLC analysis, utilizing diode-array detection (DAD) and thermospray mass spectrometry (LC–TSP-MS).

2.2. HPLC–DAD analysis

The equipment used for the HPLC–DAD analysis consisted of a HPLC HP 1090A (Hewlett-Packard, Palo Alto, CA, USA) with a diode-array detector HP 1040 managed by a Workstation HP Series 9000. A Waters-Millipore Novapak C_{18} column (Waters, Milford, USA), 150×4.6 mm, 5 μm, supplied with a 10-mm precolumn packed with the same material, was used. The eluents were: 0.16% formic acid–0.20% triethylamine in water and methanol (Optima grade). The flow-rate was adjusted to 0.9 ml/min and the injected volume of each extract was 20 μl. The effluent was monitored at 254, 280 and 360 nm and scanned between 190 and 400 nm by the diode-array detector.
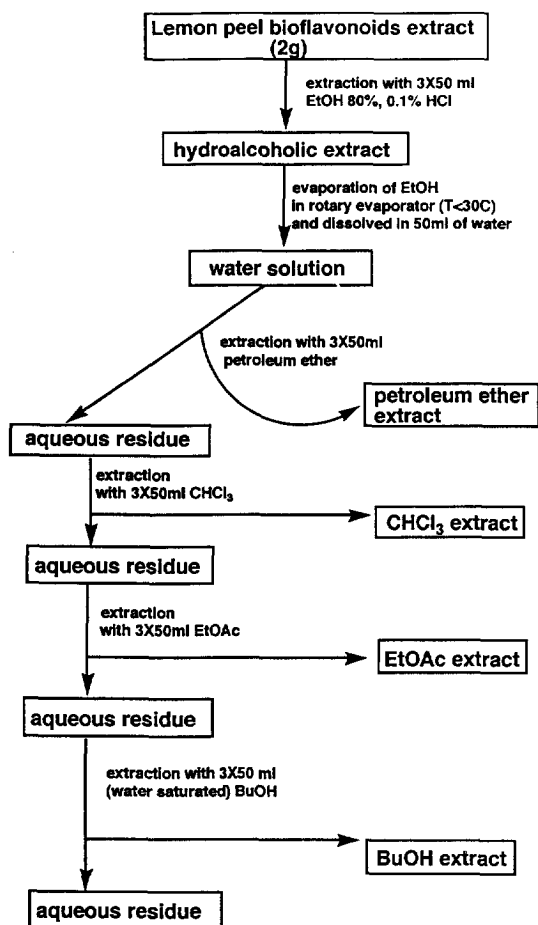
Fig. 1. Extraction and fractionation method.

2.3. HPLC–MS analysis

A HPLC Varian 9010 (Walnut Creek, CA, USA) with ABI Spectroflow 783 UV–Vis detector coupled with a Vestec Model 201 mass spectrometer was used for the HPLC–MS analysis. The interface was of the thermospray type and the mass spectrometer operated in the negative-ion thermospray discharge ionization mode. Sensitivity was enhanced using a triethylammonium formate buffer at the concentration described above for the HPLC–DAD analysis [12]. Methanol was used as organic modifier. The ion source temperature was 250°C and negative-ion spectra between 200 and 700 u were recorded at 3.2 s per scan.

2.4. Hydrolysis conditions

Acidic hydrolysis on the EtOAc extract was performed. The EtOAc extract (2 ml) was evaporated, dissolved in 6 M HCl solution and heated for 1 h at 105°C. Further extraction with EtOAc gave an extract that was analyzed by HPLC–DAD.

2.5. Antioxidative activity test

Pure lard (Hatfield Quality Meats, Hatfield, PA, USA) without any additives was used as substrate to evaluate the antioxidant activities of lemon peel extracts. The test samples were prepared by mixing the extracts with lard in 0.02% concentration on weight basis. A 670 Rancimat (Metrohm, Herisau, Switzerland) was used. A portion of 2.5 g of each test sample was loaded into the reaction vessel cylinder. Six different samples were examined in one batch: the four extracts, a sample added with BHT (butylated hydroxytoluene) and the control (only lard). The air supply was maintained at 20 ml/min and the heating temperature was kept at 100°C throughout the experiment.

3. Results and discussion

Good separation of the compounds present in the different extracts was obtained using several linear solvent gradients. Fig. 2 shows a representative chromatogram acquired using HPLC–DAD analysis. UV–Vis spectra of each peak were acquired, some of them showing the typical absorbance of the described groups of flavonoids. Both DAD and MS spectra were obtained for each compound. In this way the data obtained from the different analytical systems were compared and combined. The compounds were identified by their retention time characteristics, UV–Vis and mass spectra, specifically the structure of the aglycons was determined by HPLC–DAD.

Several flavanones, flavonols and flavones were found in the EtOAc extract of lemon peel by LC–TSP-MS. Peaks at m/z 596, 450 and 288
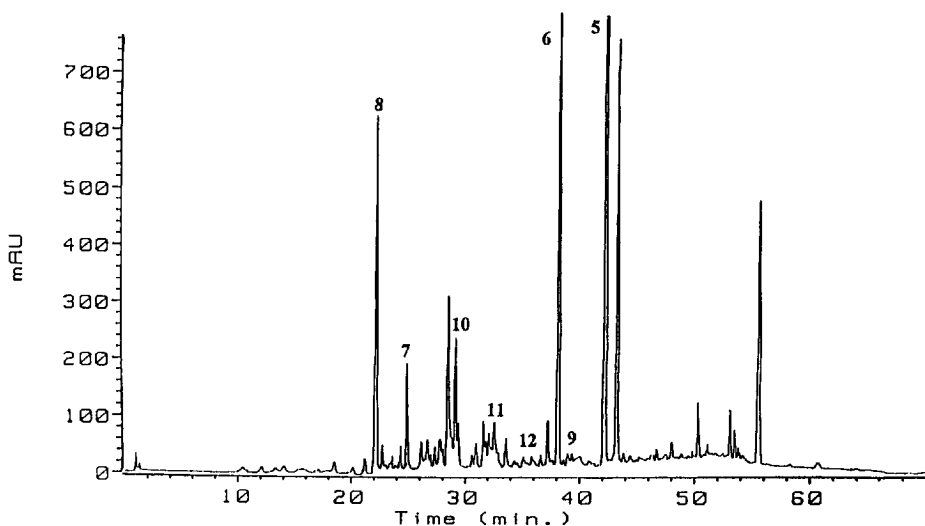
Fig. 2. Analytical HPLC separation of petroleum ether extract (254 nm).

corresponding respectively to the molecular ion [M]$^-$, the fragment resulting from the loss of a rhamnosyl moiety [M − 146]$^-$ and the fragment resulting from the loss of a further glucose moiety, another 162, were observed in the mass spectrum of the compound identified as eriodictyol-7-rutinoside (Fig. 3). The mass spec-

tra of the two other flavanones identified (hesperitin-7-rutinoside, naringenin-7-rutinoside) had similar patterns (Table 1). These three compounds showed UV–Vis spectra with a strong absorbance at 286 nm and a secondary absorbance at 328 nm, confirming the structure of the aglycones. Rutin, quercitin-3-rutinoside,
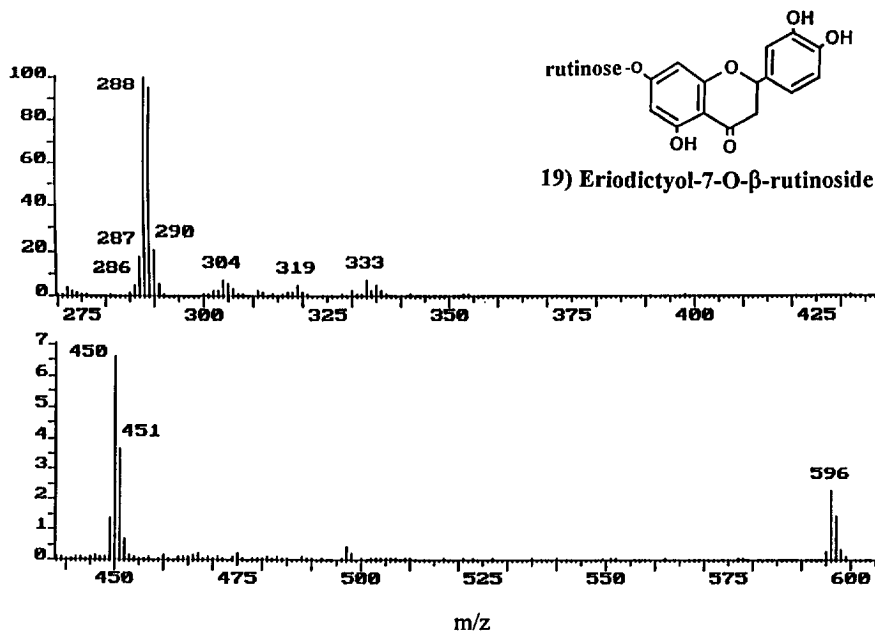

19) Eriodictyol-7-O-β-rutinoside

Fig. 3. Mass spectrum of eriodictyol-7-rutinoside.

Table 1
List of identified compounds and MS fragments

Compounds	Observed masses	UV maxima	Extract
Limonoids			
(1) Nomilinic acid	$[M-1]^- = 531$		$CHCl_3$ extract
(2) Limonin	$[M]^- = 470$	285	$CHCl_3$ extract
(3) Limonoic acid	$[M-1]^- = 487$		$CHCl_3$ extract
(4) Limonin 17-β-D-glucoside	$[M-1]^- = 649$		$CHCl_3$ extract
Phenyl propanoids			
Coumarin			
(5) Isoimperatorin	$[M]^- = 282$	312–267–250–244	petroleum ether extract
(6) Bergamottin	$[M]^- = 328$	299–263–249–243	petroleum ether extract
(7) Bergaptol	$[M]^- = 196$	313–269	petroleum ether extract
(8) Limettin derivative a	$[M]^- = 260$		petroleum ether extract
(9) Limettin derivative b	$[M]^- = 328$	324–250–247–222	petroleum ether extract
Phenyl propanoids glycosides			
(10) Citrusin A	$[M]^- = 520; [M-162]^- = 358$		petroleum ether extract
Flavonoids			
Flavones-C-glucosides			
(11) 6,8-di-C-glucopyranosyl-luteolin	$[M]^- = 642$	344–274–257	petroleum ether extract
(12) 6,8-di-C-glucopyranosyl-apigenin	$[M]^- = 626$	335–274	
Flavonols			
(13) Limocitrol	$[M]^- = 376; [M-30]^- = 346; [M-60]^- = 316$	377–350sh–275–260	EtOAc extract
(14) Isolimocitrol	$[M]^- = 376; [M-30]^- = 346; [M-60]^- = 316$	375–350sh–276–260	EtOAc extract
(15) Limocitrin	$[M]^- = 346; [M-30]^- = 316$	378–340sh–273sh–259	EtOAc extract
(16) Rutin	$[M]^- = 610; [M-146]^- = 464; [M-308]^- = 302$	361–258	EtOAc extract
Flavones-O-glucosides			
(17) Diosmetin-7-rutinoside	$[M]^- = 608; [M-308]^- = 300$	344–268–253	EtOAc extract
Flavanones			
(18) Hesperitin-7-rutinoside (hesperidin)	$[M]^- = 610; [M-146]^- = 464; [M-308]^- = 302$	330–286	EtOAc extract
(19) Eriodictyol-7-rutinoside (eriocitrin)	$[M]^- = 596; [M-146]^- = 450; [M-308]^- = 288$	330–286	EtOAc extract
(20) Naringenin-7-rutinoside	$[M]^- = 580; [M-146]^- = 434; [M-308]^- = 272$	328–284	EtOAc extract

was also present in the EtOAc extract. This flavonol showed a mass spectrum similar to hesperitin-7-rutinoside with signals at m/z 610, 464 and 302, but the UV–Vis spectrum is totally different, showing maxima at 361 and 258 nm.

Methoxylated flavonols were also found in the EtOAc extract. Signals at m/z 376 $[M]^-$, 346 $[M-30]^-$, and 316 $[M-60]^-$ corresponding to the molecular ion and to one and two losses of methoxy groups were observed in the mass spectrum of the compound identified as limocitrol (Fig. 4). The isomer isolimocitrol, whose mass spectrum showed the same signals as

above, was identified using UV–Vis spectra data [11]. Limocitrin, another polymethoxylated flavonol, showed a mass spectrum with signals at m/z 346 $[M]^-$ and 316 $[M-30]^-$. These last three compounds are the only polymethoxylated flavonols present in the lemon peel, while other citrus species show flavones with a higher number of methoxylations.

The only flavone we found in the lemon peel EtOAc extract was diosmetin-7-rutinoside, showing a mass spectrum with signals at m/z 608 and 300 corresponding respectively to the molecular ion $[M]^-$ and to the fragment resulting from
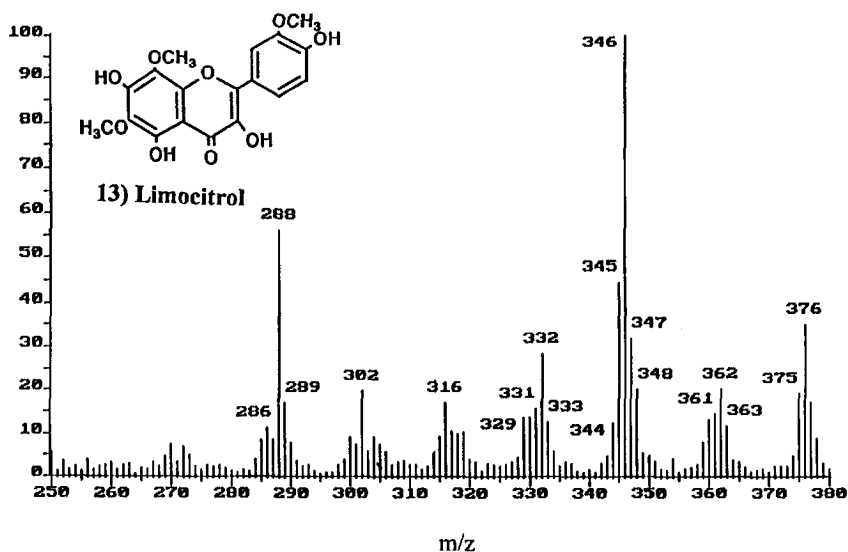
Fig. 4. Mass spectrum of limocitrol.

the loss of a rutinose moiety [M − 308]⁻. The three polymethoxylated flavonols together with quercitin, diosmetin, eriodictyol, hesperitin and naringenin, were found in the hydrolyzed EtOAc extract. Retention time characteristics, the comparison with several standards injected under the same conditions and the acquired UV–Vis spectra, allowed the confirmation of the presence of the above aglycons. Finally, the presence of two flavones-C-glucosides was presumed from the interpretation of the mass spectral data of two peaks present in the petroleum ether extract.
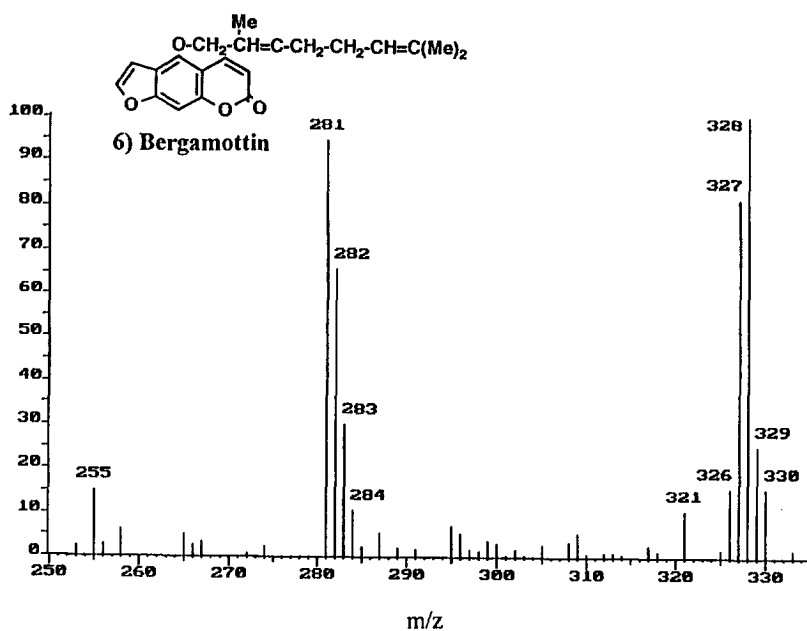

Fig. 5. Mass spectrum of bergamottin.

They show respectively a signal at m/z 642 and 626, corresponding to the molecular ions of 6,8-di-C-glucopyranosyl-luteolin and 6,8-di-C-glucopyranosyl-apigenin. The acquired UV–Vis spectra are consistent with data from the literature [11].

As shown in Table 1, flavonoids were found mainly in the EtOAc extract. The analysis of petroleum ether, chloroform and butanol extracts allowed to identify several components belonging to other groups of compounds. Particularly in the petroleum ether extract several coumarins were found according to the data in the literature [13]. These oxygen heterocyclic compounds have been investigated extensively and are useful taxonomic markers [14]. As for flavonoids, data from UV–Vis and mass spectra were used to identify the structure of each compound. Mass spectra showed the molecular ion of isoimperatorin, bergamottin, bergaptol and two limettin derivatives (Fig. 5 shows the mass spectrum of bergamottin). UV–Vis spectra were again consistent with data in the literature [13,14]. Citrusin A, a phenyl propanoid glucoside previously reported by Matsubara et al. [15], was found in the petroleum ether extract. The mass spectrum showed two main signals corresponding to the molecular ion [M]$^-$ (m/z 520) and to the loss of the glucose moiety [M − 162]$^-$ (m/z 358), corresponding to the aglycon. The structures of these compounds are shown in Fig. 6.

Several limonoids were found to be present in the chloroform extract. Limonoids are triterpene derivatives found in the *Rutaceae* family and in particular in genus *Citrus*. They are important because some give a strong bitterness to citrus juices. Among those, limonin is the main bitter compound. A compound showing a mass spectrum consistent with the structure of limonin was found in the chloroform extract. The mass spectrum showed the molecular ion [M]$^-$ (m/z 470) (Fig. 7). Limonoic acid A ring lactone, which is the equilibrium form of limonin in the fruit tissues [16], showed an [M − 1]$^-$ at m/z 487. Limonin 17-β-glucoside was also present and showed a mass spectrum with a signal at m/z 650 corresponding to the molecular ion and a weak
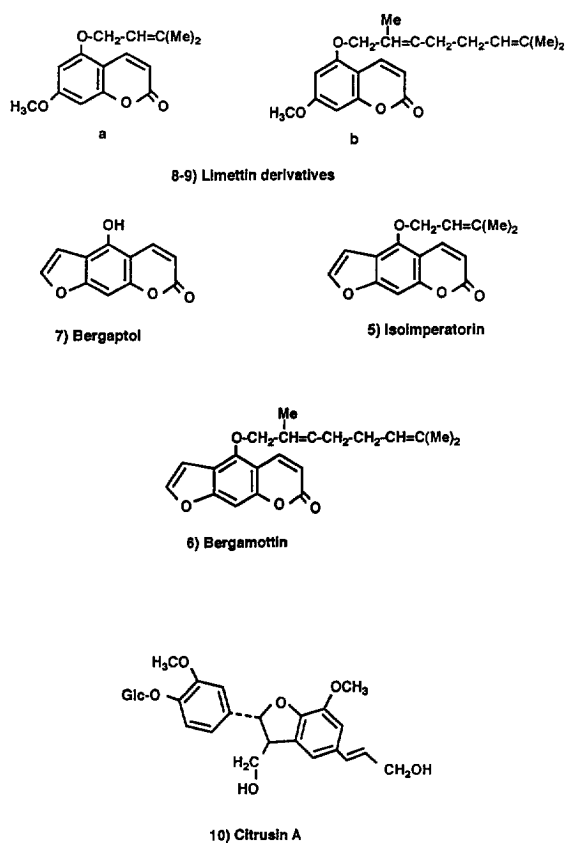

Fig. 6. Structures of identified phenyl-propanoids in the lemon peel.

signal at [M − 162]$^-$ (m/z 488) corresponding to the loss of a sugar moiety. Finally, nomilic acid gave a mass spectrum with an [M − 1]$^-$ ion at m/z 531. This data is consistent with that in the literature [16,17].

3.1. Results of antioxidant activities test

The antioxidant activities of lemon bioflavonoid extracts were measured by the Rancimat method. The induction times of lard with the extracts are shown in Table 2. Longer induction times suggest stronger antioxidant activities. Only the ethyl acetate fraction showed antioxidant activity, although the induction time is lower than that of the control sample with BHT. All other fractions did not show any antioxidant