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Analytical Methods

HPLC/DAD/MS characterisation and analysis of flavonoids and cynnamoil derivatives in four Nigerian green-leafy vegetables

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ABSTRACT

The present study sought to carry out a screening of the phenolic fraction of four Nigerian plants, *Ocimum gratissimum* L. (Og), *Vernonia amygdalina* L. (Va), *Corchorus olitorius* L., (Co) and *Manihot utilissima* Pohl. (Mu) consumed as food at least once daily by people in southwestern Nigeria and also used for medicinal purposes by local populations. HPLC/DAD and HPLC/ESI/MS analyses were applied as the most suitable techniques to investigate the phenolic content of the dried leaves. This screening allowed detection of up to 17 constituents in Va, five phenols in Mu, eight and 11 different metabolites in Co and Og, respectively. Some compounds have been detected for the first time in these vegetables: cichoric acid in Og, amentoflavone in Mu and several dicaffeoyl compounds in Va and Co. The study of the decoction demonstrated that almost all the phenolic constituents are stable even after a strong heating process such as boiling, as usually applied by Nigerian people prior to the consumption of these vegetables.

1. Introduction

Plant foods contain many bioactive compounds in addition to the identified nutrients such as proteins, lipids, vitamins, specific minerals and certain hormone precursors (Kris-Etherton et al., 2002). Humans consume and utilise a variety of vegetable materials in the form of leaves, roots, seeds and fruits. Moreover, there is growing evidence that vegetables and fruits are good sources of natural bioactive molecules, namely some vitamins, carotenoids, and phenolic compounds. Most vegetables and fruits have been reported to possess antioxidant activities which allow them to scavenge both reactive oxygen species and electrophiles, inhibit nitrosation, chelate metal ions, and modulate certain cellular enzyme activities. It has been established that part of the antioxidant activities of vegetables and fruits are related to phenolic compounds (Fraga, 2007; Halliwell, Rafter, & jenner, 2005; Rice-Evans, Miller, Bolwell, Bramley, & Pridham, 1995). Most of the compositional aspects of the vegetables, commonly used in the Western diet, are well known, nevertheless scant data are available on endemic plants from African regions. The present study attempts to improve knowledge about the phenolic compounds of the leaves of four selected vegetables commonly used for food and medicinal purposes in Nigeria. *Vernonia amygdalina* L. (Va) from the family Compositae, *Corchorus olitorius* L. (Co) belonging to Tiliaceae, *Ocimum gratissimum* L. (Og) from the Labiateae family and *Manihot utilissima* Pohl. (Mu) from Euphorbiaceae. The first three plants are mainly consumed as fresh or pot vegetable, whereas Mu is consumed mainly as tubers while the young leaves are gaining acceptance as pot vegetables.

The Vernonia leaves, called "ewuro" by the local population, are known also as "bitter leaf" and are harvested throughout the year. Regarding the phytochemical composition, a previous report on *Vernonia amygdalina* L. leaves highlighted the presence of luteolin, luteolin 7-*O*-β-glucoside and luteolin 7-*O*-β-glucuronide together with some saponins and sesquiterpene lactones as main components (Igile, Oleszek, Burda, & Jurzysta, 1995). Va is probably the most used medicinal plant in the genus Vernonia and antimalarial (Abos & Raseroka, 2003; Masaba, 2000; Oboh, 2006), antimicrobial (Akinpelu, 1999; Erasto, Grierson, & Afolayan, 2006), and anticancer activities (Izevbigie, 2003) have been documented.

O. gratissimum L., called "efinrin" by Nigerian people, is African basil and it is usually collected from May to October. With regards to its composition, within a systematic investigation on the distribution and on the intra-specific variations of vacuolar flavonoid glycosides of the genus Ocimum (Grayer et al., 2002), some data on *O. gratissimum* L. plants grown in the UK have been reported. The authors found that the profile of the main flavonoids was

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similar in all accessions belonging to the same species and they showed the main flavonoidic compounds in the leaves to be vicenin-2, luteolin 7-*O*-glucoside, quercetin 3-*O*-glucoside and quercetin 3-*O*-rutinoside. Previously, the same research group identified xanthomicrol, cirsimaritin, and kaempferol-3-*O*-rutinoside as principal flavonoids (Grayer, Kite, Abou-Zaid, & Archer, 2000). For medicinal properties, in south-western Nigeria, Og is mainly known for its antimicrobial activities against bacteria causing diarrhoea (Adebolu & Salau, 2005).

Tossa Jute (*Corchorus olitorius* L.) is an Afro-Arabian variety, quite popular for its leaves that are collected from May to December and used as a potherb. Jute leaves, also mentioned as Jew's Mallow, are popular in West Africa and the Yoruba of Nigeria call it "ewedu". It is made into a common mucilaginous soup or sauce in some West African cooking traditions. Information from a database on flavonoid contents of selected foods revealed the presence of kaempferol glycosides, rutin and isoquercitrin in *Corchorus olitorius* L. leaves (Sakakibara, Honda, Nakagawa, Ashida, & Kanazawa, 2003). In Nigeria, this plant is mainly known for its laxative activity and as a blood purifier (Aiyeloja & Bello, 2006).

Nigeria is the world's largest producer of *Manihot utilissima* Pohl. or cassava and its young leaves, known by local population as "ewe ege", are harvested throughout the year. Though less popular in the Nigerian diet compared with the previous three plants, the dietary acceptance of Mu has been increasing within local populations (Awoyinka, Abegunde, & Adewusi, 1995). It has been reported that cassava leaves have greater amounts of tannins and proteins compared to other vegetables, but no data are available on its phenolic composition.

It is estimated that these four green leafy vegetables are consumed, mainly as stew, at least once daily by the southwestern population of Nigeria.

In the present study a screening of the phenolic fraction of these vegetables was carried out by HPLC/DAD and HPLC/ESI/MS working on hydroalcoholic extracts and decoctions obtained from dried leaves. In addition, the main detected flavonoids and cynnamoyl derivatives were identified and quantified.

2. Materials and methods

2.1. Materials

All the standards used to confirm the chemical structure of some compounds (Table 1a–d) were purchased from Extrasynthese (Geney, France), with the only exception of rutin from Sigma–Aldrich (St. Louis, MO, USA).

The vegetables were harvested during the dry season in November 2006, from local farms in Akure, south-western Nigeria, and voucher specimens were deposited at the Department of Biochemistry, Federal University of Technology, Akure, Nigeria and Department of Pharmaceutical Science, University of Florence, Italy. One fresh sample of about 4 kg was collected for each Va, Co and Og, while for Mu the total fresh weight was 1.5 kg.

The samples were air-dried (5–7 days) and then oven-dried at 30 °C to constant weight. The dried samples were then kept in sealed air-tight polythene bags until analysis for a maximum of 6 months. The dried samples were finely powdered immediately before extraction. The calculated% water loss was: 92 for Va; 81 for Co; 88 for Og and 74 for Mu.

2.2. Extraction methods

2.2.1. Alcoholic and hydroalcoholic extracts

A dried sample (1 g each) was extracted with 40 ml (20 ml × 2) of MeOH or ethanol/water 7:3 (v/v) with water acidified by formic acid (pH 2.5). The samples were filtered and the clear solution directly analysed by HPLC/DAD/MS.

2.2.2. Decoction

Each dried plant sample (5 g) was boiled in water (100 ml) for 10 min. After cooling, the sample was centrifuged (5000 rpm for 10 min) and the clear solution was recovered in a conical flask, and then rinsed to 100 ml with water. This sample was analysed as such by HPLC/DAD/MS. The only exception was *Corchorus olitorius* L. that gave a highly viscous water solution (like a gel). This suggested the presence of polysaccharides, precipitated by adding an equal volume of ethanol and stirring the samples around 0 °C. The precipitate was removed by centrifugation (5000 rpm for 10 min) and the hydroalcoholic supernatant was recovered, filtered and analysed by HPLC/DAD/MS for qualitative purposes only.

2.3. HPLC/DAD/MS analysis

Analyses were performed using an HP 1100 liquid chromatograph equipped with HP DAD and 1100 MS detectors; the interface was an HP 1100 MSD API-electro spray. All the instruments were from Agilent Technology (Palo Alto, CA, USA). The MS analyses were carried out in negative mode with a fragmentor range between 80-150 V.

2.2.3. Method 1

A C12 column, 150 × 4 mm (4 μm) Synergi max® (Phenomenex-Torrance CA) maintained at 30 °C and equipped with a 10 × 4 mm pre-column of the same phase was used with a flow rate of 0.4 ml min^{-1}. The eluents were H_2O acidified to pH 3.2 by formic acid (A) and acetonitrile (B). The following linear solvent gradient was applied: from 95% A to 85% A in 5 min, to 75% A in 8 min and a plateau of 10 min, to 55% A in 12 min and a plateau of 5 min, to 10% A in 3 min, and a final plateau of 2 min to wash the column. The total time of analysis was 45 min.

2.2.4. Method 2

To improve the chromatographic performance mainly for cichoric acid, the *O. gratissimum* L. samples were analysed using a different column, particularly a Polaris-ether® (Varian) 250 × 4.6 mm, (5 μm), maintained at 30 °C. The eluents were H_2O acidified to pH 3.2 with formic acid (A) and acetonitrile (B); the flow rate was 0.8 ml min^{-1}. The following linear solvent elution method was applied: from 92% to 80% A in 10 min, to 75% A in 18 min, to 55% A in 12 min, to 5% A in 3 min, and a final plateau of 6 min to wash the column. The total time of analysis was 44 min.

2.4. NMR spectroscopy

The ^1H, ^1H-^1H COSY, and HSQC spectra of isolated nevadensin, were recorded at 300 K on a Bruker Avance-400 spectrometer operating at 400.13 MHz (14.1 T) using a 5-mm inverse probe equipped with a z-shielded gradient. The solvent used was CD_3OD, 99.8% atom D, Sigma–Aldrich.

2.5. Quantitative evaluation

The standards chlorogenic acid, rutin and luteolin 7-*O*-glucoside were used for the quantitative evaluation. Three five-point calibration curves were prepared as follows: chlorogenic acid at 330 nm (range 0.038–0.3 mg/ml and r^2 of 0.9996) was used to evaluate all the cynnamoyl compounds; luteolin 7-*O*-glucoside at 330 nm (range 0.11–0.88 mg/ml and r^2 of 0.9999) was selected to evaluate all the luteolin and apigenin derivatives, together with nevadensin; rutin at 350 nm (range 0.13–1.02 mg/ml and r^2 of 0.9999) was used to quantify all the derivatives of quercetin and kaempherol .

Table 1
List of the identified compounds by HPLC/DAD and HPLC/ESI/MS.

Peak no.	Compounds	Rt (min)	λ_{max} (nm)	$[M-H]^-$	Fragment ions	References
(a) Vernonia amygdalina L. (Va)						
Va 1	Caffeoyl quinic acid	11.4	330	353	191	
Va 2	Chlorogenic acid	12.0	330	353	191	
Va 3	Rutin	14.6	256/354	609	301	Std.
Va 4	Luteolin 7-*O*-rut.	14.7	264/348	593	285	
Va 5	Luteolin 7-*O*-glu.	15.6	260/348	447	285	Std.
Va 6	Luteolin 4′-*O*-rut.	16.1	260/336	593	285	Sakakibara et al. (2003)
Va 7 + 8	Flavonoid + caffeoyl der.	17.7	–	–	–	
Va 9	Luteolin-7-*O*-glucur.[a]	18.6	254/348	461	285	Sakakibara et al. (2003)
Va 10	1,5-Dicaffeoyl-quinic ac.	19.1	328	515	353,191	
Va 11	Dicaffeoyl-quinic ac.	19.7	328	515	335, 191, 173	
Va 12	Dicaffeoyl-quinic ac.	20.9	328	515	353; 191, 179	
Va 13	Apigenin 6-*O* or 7-*O*-glucur.	2.0	268/334	445	269	
Va 14	Luteolin	28.5	264/348	285	–	Std.
Va 15	Flavonoid	29.8	284/334	547	299; 285	
Va 16	Flavonoid[b]	34.2	336	663	269	
Va 17	Flavonoid[b]	34.9	270/336	663	531; 299	
(b) Manihot utilissima Pohl. (Mu)						
Mu 1	Rutin	14.6	356	609	300	Std.
Mu 2	Kaempferol 4′-*O*-rut.	15.6	260/348	593	285	
Mu 3	Kaempferol 3-*O*-rut.	16.2	260/348	593	285	Std.
Mu 4	Ferulic acid	19.2	330	193	179; 135	Std.
Mu 5	Amentoflavone	38.1	268/336	537		Std.

Peak no.	Compounds	Rt (min)	λ_{max} (nm)	$[M-H]^-$	Fragment ions	References	mg/g dw (SD)
(c) Corchorus olitorius L. (Co)							
Co 1	Caffeoyl quinic derivative	11.3	330	729	375, 353. 191, 179	1.0 (0.03)	
Co 2	Chlorogenic acid	12.0	326	353	191	Std.	0.19 (0.01)
Co 3	Hyperoside	15.4	256/358	463	301	Std.	2.47 (0.15)
Co 4	Isoquercitrin	15.6	256/358	463	301	Std.	2.53 (0.15)
Co 5	1,5-Dicaffeoyl quinic ac.[c]	19.1	328	515	353; 191; 179; 161		8.89 (0.49)
Co 6	Dicaffeoyl quinic ac.	19.7	326	515	353; 179; 161		
Co 7	Dicaffeoyl derivative	20.7	328	515	353; 161		3.2 (0.19)
Co 8	Quercetin derivative	22.1	354	533	505; 301		

Peak no.	Compounds	Rt (min)	λ_{max} (nm)	$[M-H]^-$	Fragment ions	References
(d) Ocimum gratissimum L. (Og)						
Og 1	Vicenin-2	11.2	270; 336	593	387	Grayer et al. (2002)
Og 2	Caffeic acid	12.3	330	179	135	Std.
Og 3	Rutin	14.6	256; 356	609	301	Std.
Og 4	Luteolin 7-*O*-gluc.	15.6	260; 348	447	285	
Og 5	Kaempferol 3-*O*-rut.	16.2	Impure	593	285	Std.
Og 6	Rosmarinic acid	19.9	328	359	197, 161, 135	Std.
Og 7	Cichoric acid	26.4	330	473	311, 179, 161, 149, 135	Std.
Og 8	Caffeoyl der.	33.8	330	–	–	
Og 9	Cirsiliol	34.3	273; 344	329		Grayer et al. (2001)
Og 10	Cirsimaritin[d]	38.8	276, 334	313	297	
Og 11	Nevadensin[e]	41.1	284; 332	343	313, 298	Grayer et al. (2001)

Abbreviations: rut. = rutinoside; glucur. = glucuronide; ac. = acid; glu. = glucoside; der. = derivative: dw = dried weight of the leaves.
[a] Coelution with a dicaffeoyl-quinic ac. derivative.
[b] Peaks showing similar UV–vis spectra.
[c] Coelututed with a quercetin glicoside.
[d] The ethanol extract from the leaves of rosemary leaves was used to confirm the identification by comparison of Rt, UV and MS spectra (Giaccherini et al., 2007).
[e] The structure was confirmed by NMR experiment on isolated compound and by comparison with literature (Grayer et al., 2001).

3. Results and discussion

3.1. Alcoholic extracts

For each plant, two different alcoholic extracts were prepared and compared. The ethanolic–water extract (7:3, v/v) was selected because it is usually applied as a nearly exhaustive extraction for flavonoids, both free or glycosilated, and for phenolic compounds from different vegetal materials (Innocenti et al., 2005; Mulinacci et al., 2004). Given that antioxidant properties have been highlighted for the methanol extracts of these plants (Akindahunsi & Salawu, 2005), these extracts were prepared to investigate their phenolic composition and to correlate the chromatographic profiles with these previous findings.

To easily compare the results, after a screening aimed at optimising the chromatographic resolution of the maximum number of the metabolites, all the samples were analysed applying the same elution method (Method 1 – Experimental section).

Comparing the methanol and ethanol–water extracts at the same wavelengths, very similar or overlapped chromatographic profiles were observed for each plant. The main differences were noticed only from the quantitative point of view. As expected, the greatest amounts of all the phenolic metabolites were in the hydroalcoholic solutions. These latter extracts were taken into account for the chemical characterisation and their chromatographic profiles at 330 nm are shown in Fig. 1 for Va (a), Mu (b), Co (c) and Fig. 2a for Og.

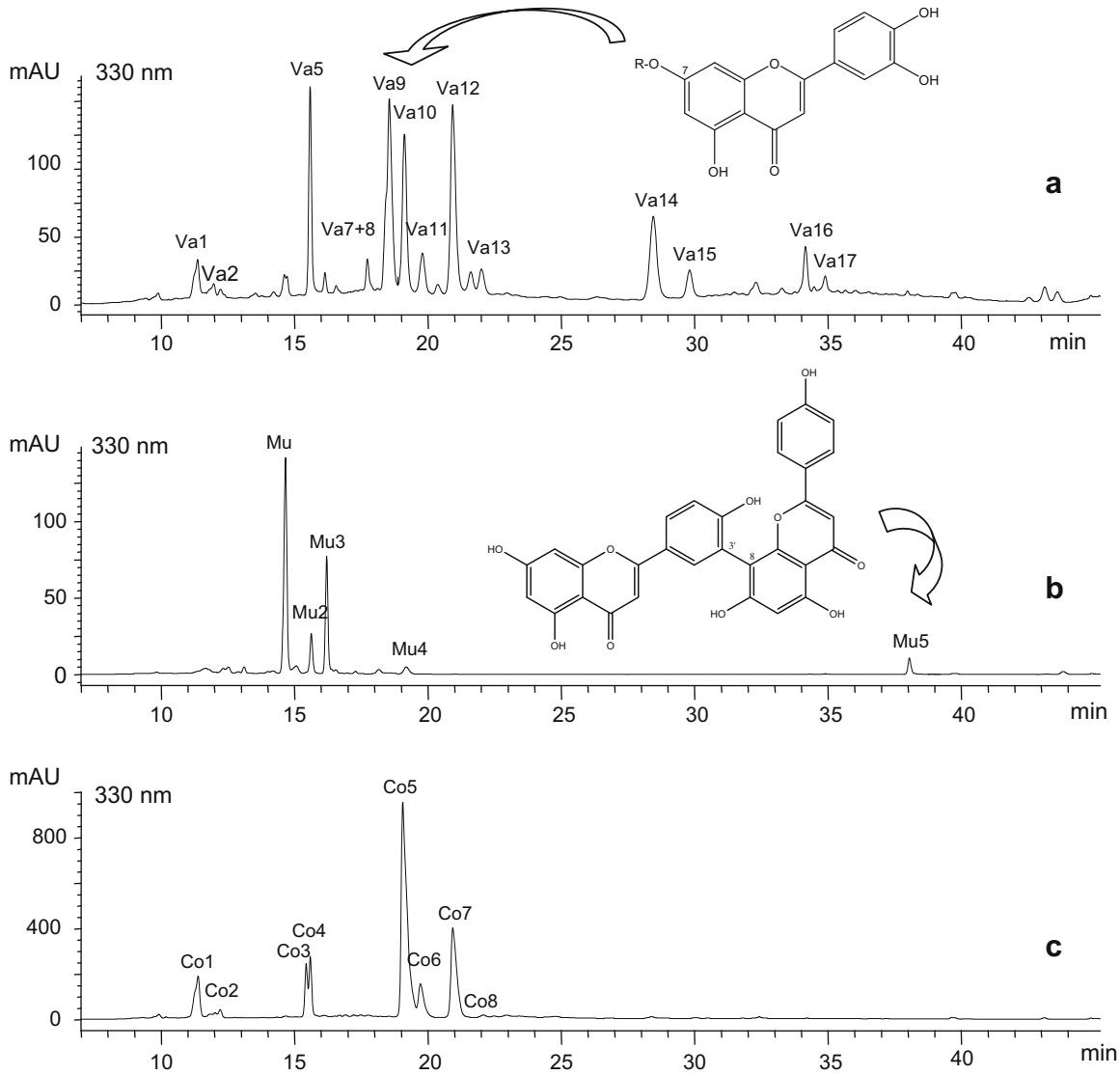

Fig. 1. Chromatographic profiles at 330 nm of the hydro alcoholic extracts: (a) *Vernonia amygdalina*; (b) *Manihot utilissima* Pohl.; (c) *Corchorus olitorius* L. at 330 nm. The profiles were obtained with the Synergi max® column and elution method 1.

3.2. Identification

The structural identification of each compound was carried out mainly on the basis of its UV–vis spectrum, retention time on reverse phase, and MS spectra obtained by applying different fragmentation energies with the API/ES technique. Moreover, the use of standard reference compounds and/or laboratory extracts helped to complete the identification. Only for nevadensin were the NMR spectra also analysed. All the identified metabolites are listed in Table 1a–d and the results are discussed for each plant below.

The main compounds detected in *Vernonia amygdalina* L. were luteolin 7-*O*-glucoside (Va 5) and luteolin 7-*O*-glucuronide (Va 9), according to previous findings (Igile et al., 1994). Moreover, from analysis of the MS spectra, two luteolin rutinosides were also detected and, according to their UV–vis spectra and literature data (Sakakibara et al., 2003), they were identified as 7-*O*-rutinoside (Va 4) and 4′-*O*-rutinoside (Va 7). As minor flavonoids, an apigenin glucuronide (Va 13) and the luteolin (Va 14) have also been detected. All these glycosides showed MS spectra with intense molecular ions together with diagnostic daughter ion at 285 m/z for luteolin and 269 m/z for apigenin. Moreover, appreciable amounts of three isomers, belonging to the class of dicaffeoyl derivatives, were found, for the first time, in Va. By comparison with a standardised artichoke extract, it was possible to exclude the presence of cynarin (1,3 isomer) and to identify the chlorogenic acid (Va 2) and the compound at Rt 19.1 min (Va 10) as 1,5 dicaffeoyl quinic acid. Three more lipophilic compounds, Va 21, Va 22 and Va 23, were characterised as flavonoids by the characteristic shapes of their UV–vis spectra.

Manihot utilissima Pohl. showed a relatively simple phenolic composition (Table 1b) with only three main derivatives (Fig. 1b), among them rutin (Mu 1) as predominant and two kaempferol glycosides (Mu 2 and Mu 3) were detected in a minor concentration. Moreover, a caffeoyl derivative (Mu 4) and a more lipophilic compound (Mu 5) were also highlighted. The latter compound, according to UV–vis and mass spectra and by comparison with the Rt of the pure standard, was identified as amentoflavone, a biflavonoid with mw of 538 Da. To the best on the authors' knowledge, this compound, which is scarcely present in green vegetables, has been detected in this plant for the first time.

As highlighted in Table 1c, several caffeoyl derivatives (Co 1; Co 2; Co 5–Co 7) were found, for the first time, as major constituents of the leaves of *Corchorus olitorius* L. Moreover, in agreement with

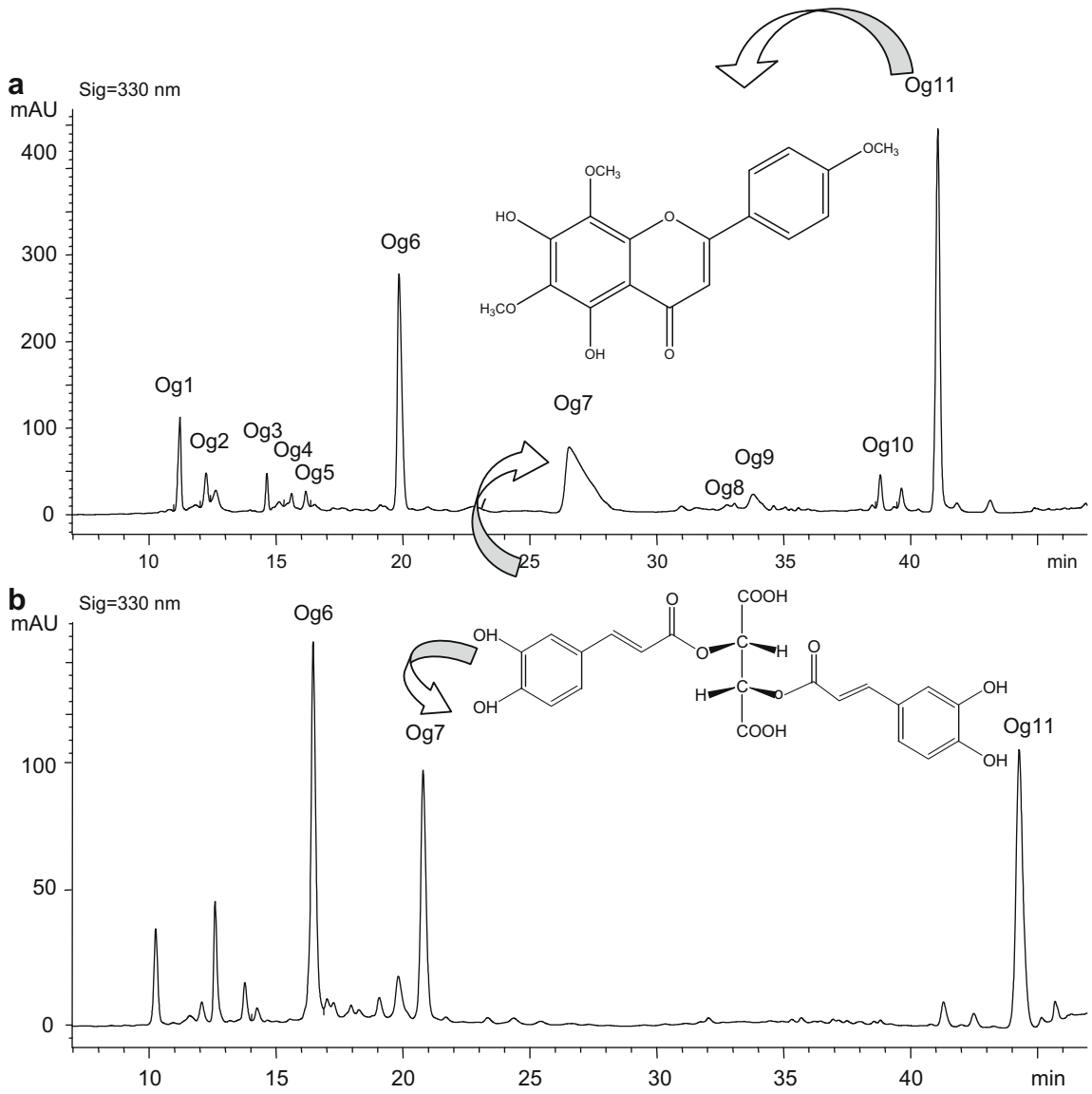

Fig. 2. Chromatographic profile at 330 nm of Og hydroalcoholic extract with: (a) Synergi max® column and elution method 1. (b) Polaris-ether column and elution method 2.

previous results (Sakakibara et al., 2003), lower quantities of hyperoside (Co 3) and isoquercitrin (Co 4) were also detected.

Due, in large part, to co-presence of metabolites belonging to several chemical classes (Table 1d), it can be said that among the four plants the chemical profile of *O. gratissimum* L. is certainly the most complex. Two caffeoyl derivatives, rosmarinic acid (Og 6) and cichoric acid (Og 7) and both confirmed by comparison with the pure standards, appeared among the major metabolites (Fig. 2a) together with nevadensin (Og 11), a lipophilic metoxyflavone at Rt 41.1 min. While the presence of rosmarinic acid in *Ocimum americanum*, *Ocimum basilicum* and *O. gratissimum* L. is well documented (Grayer et al., 2002) on the contrary, cichoric acid has not been found in the genus *Ocimum* so far. Nevadensin, recovered as pure compound by a semi preparative HPLC (data not shown), was conclusively identified by comparing the NMR data with those of a previous report (Grayer Renée, Veitch Nigel, Kite Geoffrey, Price Anna, & Kokubun, 2001). High concentrations of this metoxyflavone have already been observed in *Ocimum americanum* and *Ocimum pilosum* (Vieira, Grayer, & Paton, 2003). The structure of the most polar glycoside (Og 1) was mainly determined by the fragmentation pattern of its MS spectrum, in agreement with a previous report (Grayer et al., 2000), as vicenin 2 or apigenin 6,8-di-C-glucoside. Results from a study on the flavonoidic pattern of *O. gratissimum* L. grown in UK revealed rutin, cirsimaritin and nevadensin (Grayer et al., 2000) as the major flavonoids. From our findings on Og cultivated in the south-western part of Nigeria, these compounds (Og 3 and Og 10) were detected as minor components. In agreement with literature (Grayer et al., 2000), the presence of luteolin 7-*O*-glucoside (Og 11) was confirmed; nevertheless the kaempferol 3-*O* rutinoside, indicated by some authors as one of the major flavonoids in Og leaves (Grayer et al., 2002) was found only in traces. Nevadensin, well known as one of the more abundant external metoxy flavonoids only of *Ocimum americanum* (Grayer Renée et al., 2001; Vieira et al., 2003) but not of Og (Grayer et al., 2000, 2002), was in high concentration in our sample, as clearly emerges from Fig. 2 and Table 2.

As previously highlighted for chicory (Mulinacci et al., 2001), an anomalous shape of the peak, corresponding to the cichoric acid, was observed for the Og extract analysed using a Synergy column (Fig. 2a). Differently from other phenolic constituents, this dicaffeoyl tartaric acid was particularly sensitive to the physical-chemical characteristic of the reverse phase, showing t_R values notably modified depending on the selected HPLC column. More-

Table 2
List of identified phenolic compounds for Va, Mu and Og extracts. The data are a mean of three extracts from the same lot and the compounds are progressively numbered within each plant.

	Compound	mg/g dried weight	
		EtOH/H_2O extract (mean ± SD)	Hot water extract (mean ± SD)
Va 1	Caffeoyl quinic acid	0.122 ± 0.017	0.189 ± 0.037
Va 2	Chlorogenic acid	0.029 ± 0.001	0.169 ± 0.028
Va-*x* + *y*	Caffeoyl quinic acids	Traces	0.322 ± 0.137
Va 3	Rutin	0.157 ± 0.012	nd
Va 5	Luteolin 7-*O*-glu.	0.359 ± 0.004	0.345 ± 0.028
Va 6	Luteolin 4′-*O*-rut.	0.025 ± 0.003	nd
Va 7	Flavonoid	0.094 ± 0.004	nd
Va *z*	Caffeoyl der.	0.208 ± 0.023	nd
Va 9	Luteolin 7-*O*-glucur.	0.471 ± 0.021	0.919 ± 0.083
Va 10	1,5-Dicaffeoyl quinic acid	0.409 ± 0.006	0.227 ± 0.077
Va 11	Dicaffeoyl quinic acid	0.144 ± 0.001	0.32 ± 0.116
Va 12	Dicaffeoyl quinic acid	0.638 ± 0.009	0.224 ± 0.081
Va 13	Apigenin-*O*-glucur.	0.082 ± 0.015	0.124 ± 0.01
Va 14	Luteolin	0.425 ± 0.005	0.131 ± 0.012
Va 15–17	Others flavonoids	0.182 ± 0.004	nd
Mu 1	Rutin	15.44 ± 1.85	5.765 ± 0.065
Mu 2	Kaempferol 4′-*O*-rut.	2.21 ± 0.19	0.861 ± 0.021
Mu 3	Kaempferol 3-*O*-rut.	6.66 ± 0.50	2.3 ± 0.08
Mu 4	Ferulic acid	Traces	0.49 ± 0.07
Mu 5	Amentoflavone	0.97 ± 0.03	nd
Og 1	Vicenin-2	0.65 ± 0.04	0.706 ± 0.004
Og 2	Caffeic acid	0.25 ± 0.05	0.595 ± 0.017
Og 3	Rutin	0.39 ± 0.02	0.204 ± 0.024
Og 4	Kaempferol 4′-*O*-rut.	0.04 ± 0.01	nd
Og 6	Rosmarinic acid	1.92 ± 0.1	0.714 ± 0.126
Og 7	Cichoric acid	2.55 ± 0.14	1.75 ± 0.09
Og 10	Cirsimaritin	0.18 ± 0.01	nd
Og 11	Nevadensin	2.73 ± 0.09	0.18 ± 0.016

over, using this Synergy column, a higher concentration of cichoric acid resulted in an increase of the chromatographic peak's width and a consistent increment of the peak's asymmetry. This behaviour has been related to the more stable spatial conformation of cichoric acid with the two aromatic groups facing toward each other on the same part of the molecule (Mulinacci et al., 2001).

With the aim of optimising the chromatographic response for this dicaffeoyl tartaric acid, an RP 18 Polaris-ether column was chosen for the analysis of the Og extracts. The use of this column, with an elution method very similar to that applied on the Synergi Max column (Methods 2 and 1, respectively), allowed good symmetry of the chromatographic peak for all the tested concentrations (Fig. 2b). This analytical approach caused an inversion of the elution times for the two main caffeoyl derivatives, cichoric and rosmarinic acids (Fig. 2), and it appears more reliable for detection of trace amounts of this metabolite in the sample.

3.3. Decoctions

In Nigeria several green vegetables, among them Va, Mu, Co and Og, are usually blanched by either hot water or steam before consumption, and often indigenous people apply this practice to reduce the bitterness and/or acidity of the plant. In this context, it was considered of interest to evaluate the phenolic amount extracted after a decoction procedure that simulates the common domestic cooking process.

The HPLC profiles obtained from the decoctions were very similar to those from the hydroalcoholic extract, thus suggesting that all the main phenolic compounds remained unaltered after the hot treatment and that appreciable amounts of these metabolites remain in the broths.

Among the four plants, Va showed the greatest differences between cold and hot extracts: the luteolin 7-*O*-glucuronide (Va 5) and three polar monocaffeoyl derivatives (Va 1, Va 2 and Va $-x+y$ in Table 2) were more efficiently extracted by applying decoction rather than hydroalcoholic extraction. Regarding Mu, the chromatographic profiles of the hydroalcoholic extract almost completely overlapped the profile of the hot water extract and an analogous behaviour was highlighted also for Og. As expected, mainly due to its lipophilic nature, a consistent decrease of nevadensin was observed in the hot water extract compared to the hydroalcoholic samples.

The decoction of Co produced a broth similar to a gel, hence indicating the presence of a polysaccharide fraction, in agreement with previous findings by Japanese researchers (Yamazaki, Kurita, & Matsumura, 2008; Yamazaki, Murakami, & Kurita, 2005). To obtain a sample suitable for HPLC/DAD/MS analysis, precipitation of the polysaccharides was induced as described by Sendl, Mulinacci, Vincieri, and Wagner (1993), adding ethanol to the refrigerated decoct. After centrifugation, the supernatant analysed by HPLC/DAD, employing method 1, showed a very low concentration of phenolic constituents. Presumably they remained partially entrapped in the gel. In light of these results, the quantitative content of the phenolic fraction in the decoction of Co was not determined.

3.4. Quantitative estimation of the phenolic fraction

Evaluation and expression of the quantitative results for a complex matrix, such as an herbal extract having the co-presence of several different structures, are often neither easy nor univocal. In this case, for the quantitative evaluation, we arbitrarily chose only three reference compounds which are representative, easy to find on the market and not too expensive.

The determination was carried out both on the hydroalcoholic extracts and on the decoctions, and the results have been expressed as mg/g dried weight of the leaves (Table 2).

Generally, hot water was a good solvent to extract some of the phenolic compounds and particularly it was more efficient for simple phenols such as caffeic and chlorogenic acids (see Og and Va). All the rutinoside derivatives were better extracted with the hydroalcoholic solution, as revealed from comparison of the quantitative results of Va, Mu and Og; on the contrary, the apigenin and luteolin glucuronides from Va are in higher concentration in the decoctions than in the hydroalcoholic samples.

As regards Va, even if the extractive yields in terms of total phenols were higher for the hydroalcoholic extracts compared to the decoction, appreciable amounts of these metabolites were also present in the water broth.

Quantitative evaluation of the compounds in Mu hydroalcoholic extracts revealed that rutin was the most abundant glycoside with 15.4 mg/g dw, corresponding to 61% of all the quantified phenols, while the two kaempferol rutinosides were estimated as 2.2 and 6.7 mg/g dw, respectively. A low concentration of the amentoflavone was measured in the alcoholic sample and, as expected, this lipophilic molecule was completely absent in the hot water extract.

The quantitative estimation of the metabolites in the alcoholic extract of Og revealed cichoric acid (2.55 mg/g dw), rosmarinic acid (1.92 mg/g dw) and nevadensin (2.73 mg/g dw) as dominant compounds. For this plant, hot water appeared to be less efficient in the extraction of these major phenols, however the amount of total phenols detected in the broth (207.9 ± 13.8 mg/l) was comparable to that obtained for Va.

Regarding Co, only the phenols from the hydroalcoholic extract were quantitatively evaluated (Table 1c) while the highly viscous broth was not considered due to the very low content of these metabolites. The dominant compounds for this plant were dicaffeoyl quinic acids (12.1 mg/g dw) and two quercetin monoglycosides (up to 5 mg/g dw). It must be taken into account that this plant is mostly consumed by Nigerian people as a whole, without

removing the boiling broth; therefore it can be considered a good source of dietary phenols together with appreciable amounts of fiber (Yamazaki et al., 2005, 2008).

In particular the total amounts of phenols in the broths were 148.5 ± 30 mg/l, 474.7 ± 12.8 mg/l and 207.5 ± 13.8 mg/l for Va, Mu and OG, respectively.

4. Conclusions

Screening of the phenolic content of these plants allowed detection of up to 17 constituents in Va, five phenols in Mu, eight and 11 different metabolites in Co and Og, respectively. Some compounds have been detected for the first time in these vegetables: among them cichoric acid in Og, amentoflavone in Mu and several dicaffeoyl compounds in Va and Co. The most complex chromatographic profile was obtained for Og, while the simplest one was evidenced for Mu which resulted also the richest plant in terms of total phenols for each gram of dried leaves.

The study of the composition of the decoction demonstrated that almost all the phenolic constituents are stable even after a strong heating process such as boiling, the typical preparation method applied by Nigerian people prior to consuming these vegetables. A consistent part of these phenolic constituents, mainly the glucuronides, were efficiently extracted with hot water with a consequent reduction of their content in the blanched plant. Nevertheless, due to the presence of several phenols known as bioactive metabolites healthy for humans, a re-utilisation of these broths should be encouraged in domestic activities.

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