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Analytical Methods

Identification and quantification of polyphenolic compounds from okra seeds and skins

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ABSTRACT

The aims of the present work were to identify and quantify the polyphenolic profile of okra (*Abelmoschus esculentus*), a vegetable almost worldwide consumed. Since the knowledge about the okra polyphenolic compounds is limited, the seeds and the skins of okra were separately analyzed. The seeds, which represent the 17% of the vegetable and are richer in phenolic compounds, were mainly composed by oligomeric catechins (2.5 mg/g of seeds) and flavonol derivatives (3.4 mg/g of seeds). The skins polyphenolic profile was composed principally by hydroxycinnamic and quercetin derivatives (0.2 and 0.3 mg/g of skins). These findings in associations with the high content of okra in carbohydrates and proteins enhance the importance of this foodstuff in the human diet.

1. Introduction

Okra, *Abelmoschus esculentus* (L.) *Moench*, also known as lady's finger, bhindi and gumbo; is a flowering plant valued for its edible fruits and belongs to the family of Malvaceae. The production in 2004 was more than 4.5 milions of tons in more than 33 countries with India in top, producing 3.5 million tons (FAOSTAT). Okra is of African origin where it has been cultivated for more than 4000 years but now is also grown in different tropical and warm temperature regions of the world, like Greece, Iran, Egypt, India, Japan, southern United States and Turkey, Philippines (Karakoltsidis & Constantinides, 1975). The fruit is a greenish capsule up to 20 cm long, slightly curved, six-chambered pod of fibrous texture, containing numerous seeds (Karakoltsidis & Constantinides, 1975; Lengsfeld, Titgemeyer, Faller, & Hensel, 2004). Okra was used as a mucilaginous food additive against gastric irritative and inflammative diseases, due to its high content in carbohydrates. The anti-adhesive qualities of okra were assumed to be due to a combination of glycoproteins and highly acidic sugar compounds making up a complex three-dimensional structure (Lengsfeld, Faller, & Hensel, 2007; Lengsfeld et al., 2004; Wittschier et al., 2007). The seeds are found to be, as soybeans, a high-protein source due to its high lysine level and for that reason they could used as a supplement to cereal based diets in which lysine is the first limiting amino acid (Al-Wandawi, 1983; Karakoltsidis & Constantinides, 1975).

As far as okras chemical composition, different studies have been reported. K, Na, Mg and Ca were found to be the principal elements, with Fe, Zn, Mn and Ni to be also present (Al-Wandawi, 1983; Karakoltsidis & Constantinides, 1975; Moyin-Jesu, 2007). The quantitative analysis of the total dietary fibre, protein, fat, mineral, ash and carbohydrate is thoroughly reported (Al-Wandawi, 1983; Deters, Lengsfeld, & Hensel, 2005; Kahlon, Chapman, & Smith, 2007; Karakoltsidis & Constantinides, 1975; Lengsfeld et al., 2004, 2007; Moyin-Jesu, 2007; Wittschier et al., 2007). The volatile fraction of okras volatile components has also been studied (Ames & Macleod, 1990; Camciuc, Bessiere, Vilarem, & Gaset, 1998).

However, the data is limited in regard of okras polyphenolic profile identification and qualitative analysis, since only four quercetin derivatives were just recently identified (Huang, Wang, Eaves, Shikany, & Pace, 2007; Shui & Peng 2004).

Besides, phenolic compounds are widely distributed in fruits and vegetables and have great importance in the nutritional, organoleptic and commercial properties of these fruits and their derived products through their contributions to sensory-attributes of these fruits (color, bitterness and astringency) (Harborne, 1986; Harborne & Williams, 2000; Havsteen, 2002). Epidemiological studies have indicated that regular consumption of foods rich in phenolic compounds is associated with reduced risk of cardiovascular diseases, neuro-degenerative diseases, and certain cancers (Haslam, 1996; Havsteen, 2002; Romani et al., 2005; Steinmetz & Potter, 1996). The qualitative and quantitative determination of flavonoids and phenolic acids derivatives in natural matrices is a difficult task even for modern analytical techniques, and the use

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of the HPLC coupled with diode array detection and mass spectrometry techniques seems to give the fastest and most robust answers (Arapitsas, Menichetti, Vincieri, & Romani, 2007; Arapitsas, Sjöberg, & Turner, 2008a; Arapitsas & Turner, 2008b; de Rijke et al., 2006; Maatta-Riihinen, Kamal-Eldin, & Torronen, 2004).

The objectives of this work were the separation, identification and quantification of seeds and skins okra different classes of phenolic compounds. For these puroposes a modern HPLC system was used, which comprised of a 1.8 μm C18 column to provide high resolution separation in short time and with low solvent consumption. HPLC was coupled both with DAD and ESI/MS/MS detector to facilitate the identification of the different classes by combining the information acquired from the UV/Vis spectra of the fragmentation pattern from the triple quadruple mass spectrometer. Diode array detector was also employed to furnish, for the first time in literature, data for the quantitative analysis of this important foodstuff in human diet.

2. Materials and methods

2.1. Standards and solvents

Caffeic acid, sinapic acid, ferulic acid, gallic acid, *p*-coumaric acid, 3,4-dihydroxy-benzoic acid, *p*-hydroxy-benzoic acid, rosmarinic acid, catechin, epicatechin, epigallocatechin, epigallocatechin gallate and chlorogenic acid were obtained from Fluka Chemie AG (Switzerland). Cynarin (1,3-dicaffeoylquinic acid) was supplied by Roth (Germany). The pure flavonoid compounds, luteolin 7-*O*-glucoside and luteolin 7-*O*-rutinoside, luteolin, quercetin, quercitrin (quercetin 3-*O*-rhamnoside), rutin (quercetin 3-*O*-rutinoside), isoquercitrin (quercetin 3-*O*-glucoside), kaempferol, kaempferol-3-glucoside, kampferol-3-rutinoside, isorhamnetin 3-rutinoside, isovitexin, vitexin, vitexin 2″-*O*-rhamnoside, morin, miricitrin, verbascoside, and apigenin 7-*O*-glucoside were purchased from Extrasynthese S.A. (Lyon, Nord-Genay, France). All solvents were HPLC grade and were obtained from Merck (Darmstadt, Germany).

2.2. Sample preparation

A typical extraction procedure for vegetable phenolic compounds was followed (Innocenti et al., 2007; Romani, Vignolini, Isolani, Ieri, & Heimler, 2006). Three portions of 100 g okra were, separately, divided into seeds (17.3 g ± 0.3) and skins (82.7 g ± 0.5), lyophilized to obtain 15.5 g skins (±0.2) and 2.1 g seeds (±0.1), homogenized, and extracted separately with 2 × 100 mL 70% v/v ethanol adjusted to pH 3.2 with formic acid, at room temperature, under stirring, for 2 × 3 h. The 3 seeds and 3 skins extracts were completely defatted with 3 × 50 mL *n*-hexane, then concentrated under vacuum (Rotavapor 144 R, Büchi, Switzerland), dissolved with the extraction solvent to a final volume of 10 mL, filtered (0.45 μm) and directly analyzed by HPLC/DAD and HPLC/MS.

2.3. HPLC analysis

HPLC/DAD analyses were carried out using a HP 1100L liquid chromatograph equipped with a DAD detector (Agilent Technologies, California, USA). The column used was a 1.8 μm Zorbax SB-C18, 100 × 2.1 mm i.d. (Agilent, Palo Alto, CA), operating at 27 °C and with a flow rate of 0.2 mL/min. The injected volume was 3 μL. The mobile phase was a multi-step linear solvent gradient system consisting of (A) H_2O (adjusted to pH 3.2 by HCOOH) and (B) CH_3CN. The elution profile was: 3 min 97% A, then the solvent B was increased first to 15% in 2 min, to 18% in 7 min, to 30% in 8 min, to 95% in 4 min, constant for 4 min and subsequently decreased to 3% in 2 min.

HPLC/MS analyses were performed by connecting to the HPLC system to a triple quadruple mass spectrometer (Applied Biosystems, MDS Sciex. Toronto, Canada) equipped with a pneumatically assisted ESI interface. Initial optimization of the parameters for the ESI/MS and ESI/MS/MS analyses of the standards and the total extract was performed by direct infusion into the ES ionisation source. For the direct infusion optimization the standards were dissolved in acetonitrile:water:formic acid (20:79:1 v:v:v) (10 μg/mL) and the total extract was diluted 1/100 with the same solution. Both samples, standards and total extract, were infused into the ES ionisation source using a syringe pump at a flow rate 10 μL/min. The optimum conditions found for the analysis of anthocyanins were: negative ion scan mode, nebuliser gas (NEB) 0.69 bar, curtain gas (CUR) 0.48 bar, collision gas (CAD) 5×10^{-5} Torr, ion spray voltage (IS) −3500 V, source temperature (TEM) 350 °C, focusing potential (FP) −250 V, entrance potential (EP) −10 V, declustering potential (DP) −35 V, collision energy (CE) −45 V, collision cell exit potential (CXP) −10 V. Nitrogen gas was used for CUR, CAD and NEB. The enhanced MS scan mode (EMS) and the enhanced product ion scan were performed in mass range of 150–1500 u. The operating system was Analyst 1.4.

2.4. Identification analysis

Compounds were identified by comparing their retention times and spectra to those of standards, when available. Identification of peaks was then confirmed by spiking samples with standard mixtures. Unknown chromatographic peaks were tentatively identified via their spectral features and by literature data.

2.5. Quantitative analysis

Quantification of individual compounds was performed by HPLC/DAD using a five point regression curve ($r^2 > 0.998$) in the range of 5–500 μg/mL. For compounds that standard was available their quantification was performed by calibration curve of the corresponding standard. Where that was not possible, hydroxylcinnamic derivatives amounts were calculated at 330 nm using the caffeic acid standard calibration curve, catechin derivatives at 280 nm using the catechin calibration curve and flavonols at 350 nm using the rutin calibration curve. Total amounts were calculated as catechin for catechins, rutin for flavonols and hydroxycinnamic derivatives as caffeic acid. Precision/injection repeatability test (expressed as relative standard deviation, RSD, in%) was performed by six ($n = 6$) replicate injections of rutin, catechin and caffeic acid standard solutions at a concentration of 50 μg/mL. These tests showed good precision in peak area (RSD <2%) and peak retention time ±2%.

3. Results and discussion

The main interests of this research project were the identification and quantification of the polyphenolic compound present in the seeds and the skins of okra. As far as authors' knowledge is concerning this is the first work about the identification of the different polyphenolic compounds present in okra seeds and skins and their quantification. The hyphenation use of the DAD and triple quadruple MS detector gave the possibility of the simultaneous analysis of different polyphenolic classes.

In the Figs. 1 and 2 the chromatographic profile of okra skins and seeds are reported at different wavelengths. The use of the DAD detector offers the possibility to analyze and quantify different polyphenolic classes. Catechins have characteristic UV/Vis spectra, with maximum absorbance at 280 nm, and no absorption

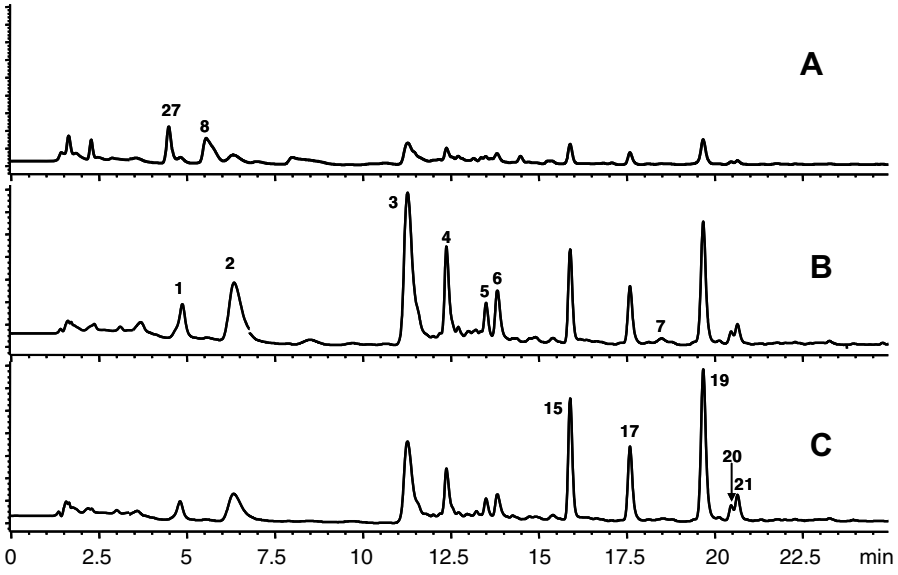

Fig. 1. HPLC/DAD chromatogram of okra skin extract reported at (A) 280 nm, (B) 330 nm and (C) 350 nm.

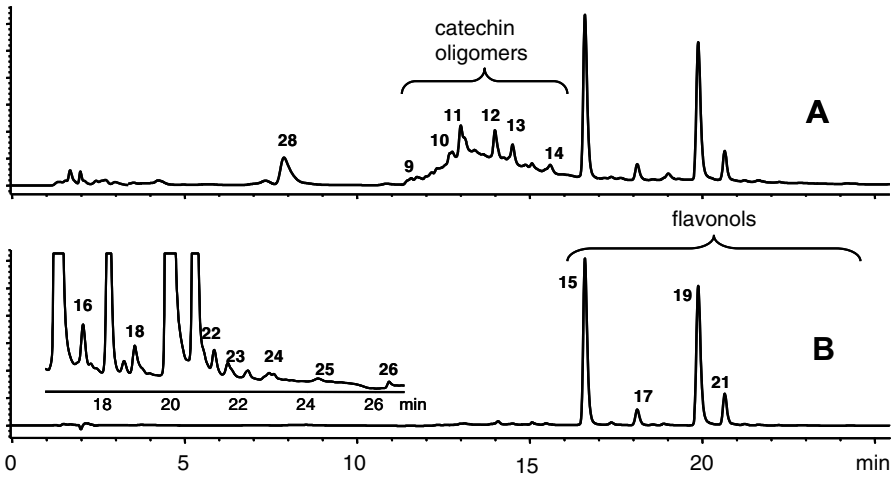

Fig. 2. HPLC/DAD chromatogram of okra seed extract reported at (A) 280 nm and (B) 350 nm.

at 330 or 350 nm, are clearly detected and quantified at 280 nm (Figs. 1A, 2A and 3A). Derivatives of the hydroxycinnamic acid also have characteristic UV/Vis spectrum, with λ_{max} between 310 and 330 nm and are generally quantified at 330 nm (Fig. 1B and 3B). Finally also flavonols have characteristic UV/Vis spectra, with λ_{max} between 340 and 370 nm and are generally quantified at 350 nm (Fig. 1C, 2B, 3C and 3D) (Innocenti et al., 2007; Romani et al., 2004, 2006). Although the spectral information provided by the DAD detector are useful for the identification of the chromophoric part(s) of the polyphenolic analyte(s) it is insufficient for the structural identification (Arapitsas et al., 2007; Arapitsas, Sjöberg, & Turner, 2008a; Arapitsas & Turner, 2008b; de Rijke et al., 2006; Romani et al., 2006).

Tandem mass spectrometry (MS/MS), and in particular production analysis, which acquires mass spectra from product ions produced from a selected precursor ion can provide further information for more accurate identification analysis.

As shown in Table 1 the combination of the spectral data from a DAD detector and a tandem mass spectrometer gave the possibility of the tentative identification of different polyphenolic classes present in okra seeds and skins.

The identification of all flavonols, except of peaks 17 and 20–22, was also confirmed by the commercial standards. The major peak number were quercetin derivatives (peaks 15, 17–19, 21, 22 and 26) with miricitrin (peaks 16 and 24), isorhamnetin (peak 20) and keampferol (peak 23 and 25) derivatives to be less abundant. The data present in Table 1 shown that okra seeds have a bigger number of flavonols compared to the skin.

The second class of flavonoids found was catechins. Also in that case seeds were having a major number of catechins. Furthermore, seeds catechins appear to be more complicated and with larger molecular weight since dimeric, trimeric and tetrameric forms of epigallocatechin were detected.

Shui & Peng (2004) have isolated and NMR characterized four phenolic compounds from okra: quercetin 3-*O*-glucosyl (1‴ → 6″) glucoside 15, quercetin 3-*O*-xylosyl (1‴ → 2") glucoside 17, quercetin 3-*O*-glucoside 19 and quercetin 3-*O*- (6′-*O*-malonyl) glucoside 21. As far as our knowledge goes, this is the only other work regarding the identification of okra phenolic compounds.

Okra skins which represent the biggest part of the vegetable (82.7%), were found to be rich in hydrocinnamic acid derivatives, a polyphenolic group that was absent from the seeds. Even though

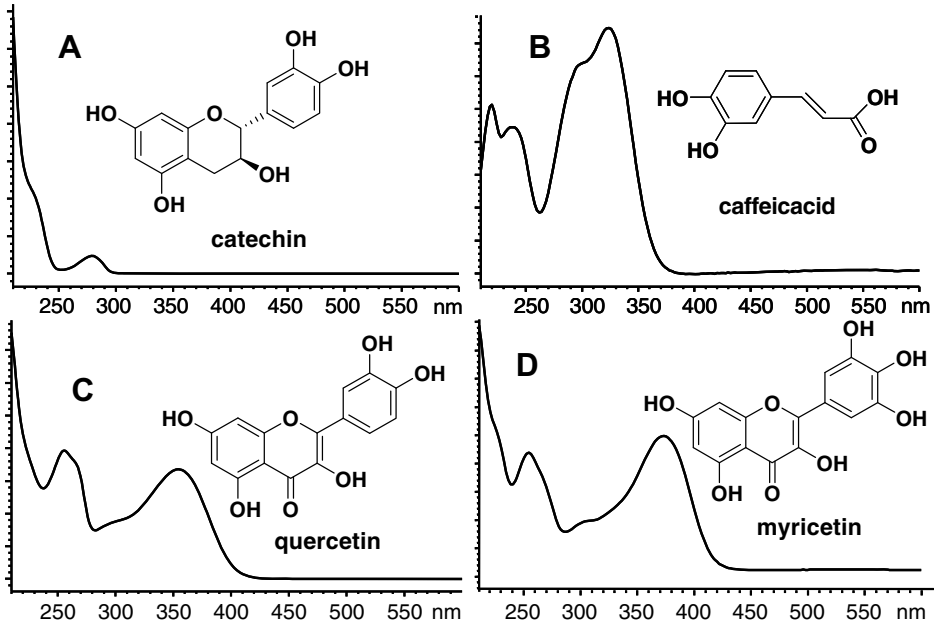

Fig. 3. Typical UV/Vis spectra of the different phenolic classes identified, where (A) is representative UV/Vis spectrum for oligomeric catechins; (B) for hydroxycinnamic derivatives and (C and D) for flavonols.

Table 1
Chromatographic and spectroscopic properties and quantitative results of okra seeds and skins polyphenolic compounds (Figs. 1 and 2)

Peak	t_R (min)	λ_{min} (nm)	MS^- (m/z)	MS/MS (m/z)	Quantitative analysis			Tentative identification
					Okra (μg/g)	Skins (μg/g)	Seeds (μg/g)	
Hydroxycinnamic derivatives (Fig. 1B)								
1	4.8	328	326	163, 147	16.24*	19.64*		*p*-coumaroyl-hex
2	6.3	326	385	193, 177	38.04*	46.00*		Sinapoyl-hex
3	11.3	326	385	193, 177	67.28*	81.35*		Sinapoyl-hex
4	12.4	326	399	193, 207	24.74*	29.92*		Sinapoyl-feruloyl
5	13.5	326	387	223, 207, 193, 177	9.74**	11.78**		Sinapoyl-feruloyl
6	13.8	326	207		14.84**	17.95**		Sinapoyl derivative
7	18.5	320	ND	ND	3.87***	4.68***		Cinnamic derivative
Total					174.29*	211.32*		
Oligomeric catechins (Fig. 1A and 2A)								
8	5.6	278	ND	ND	122.17**	147.73**		Catechin derivative
9	11.6	280	609	305, 125				Epigallocat dimer
10	12.3	280	913	609, 305				Epigallocat trimmer
11	12.7	280	1217	913. 609, 305				Epigallocat tetramer
12	13.0	280	609	305				Epigallocat dimer
13	14.5	280	609	305				Epigallocat dimer
14	16.0	280	579	289				Catechin dimer
Total[a]					557.86**	147.73**	2518.44**	
Flavonols (Fig. 1C and 2B)								
15[b]	16.2	356	625	301, 177	363.41*	93.50*	1653.72*	Querc 3-*O*-diglu
16[b]	17.4	356	479	317, 287, 271	3.96**		21.36**	Miric 3-*O*-glu
17	17.7	356	595	301	86.72*	69.56*	168.72*	Querc 3-*O*-glu-xyl
18[b]	18.9	356	609	463, 301, 285, 177	3.92***		22.68***	Querc 3-*O*-rut
19[b]	19.7	356	463	317, 285	320.95*	117.44*	1176.36*	Querc 3-*O*-glu
20	20.5	356	609	477, 315, 299	11.13**	13.46**		Isorhamn 3-*O*-glu-pent
21	20.6	356	549	505, 301	72.47*	26.18**	293.76*	Querc 3-*O*-(malonyl)glu
22	21.2	356	463	317	2.97***		17.16***	Miric 3-*O*-rhm
23[b]	21.6	355	447	285	2.03***		11.76***	Kaempf 3-*O*-glu
24[b]	22.9	330	317		1.29***		7.44***	Miric
25[b]	24.4	355	285		ND***		ND***	Keampf
26[b]	26.5	371	301		0.69***		3.96***	Querc
Total					798.93**	320.14**	3387.12**	
Unknown compounds								
27	4.5	260	326	161				
28	7.9	260	323	203				

Abbreviations: hex: hexose, epigallocat: epigallocatechin, querc: quercetin, miric: miricitrin, glu: glucose, xyl: xylose, rut: rutinoside, pent: pentose, isorhamn: isorhamnetin, kaempf: kaempferol.
(*) <1%, (**) <2% and (***) <5% Standard deviation.
[a] Only the total amount of oligomeric catechins is given for okra seeds.
[b] Identification and quantification made by using standard.

a large number of the hydroxycinnamic commercial available standard was used in the identification analysis, none of them was detected in the extract. The tentative identification based on the UV/Vis spectra and the tandem MS fragmentation patterns of each peak reveal the presence of compounds having *p*-coumaric, sinapic and ferulic acid.

The quantitative analysis of seeds and skins have pointed out that flavonols is the major polyphenolic class in both parts of the plant and consequently in total. The second most abundant class for the entire vegetable and its seeds were catechins and hydroxycinnamic derivatives for the skins. Seeds were found to have more than 10 times higher concentration in flavonols and almost 15 times higher concentration in catechins compared to skins but skins were the only part of the vegetable containing hydroxycinnamic acid. The chromatographic difficulties for the separation of the seeds oligomeric catechins resulted in low chromatographic resolution and so these compounds were quantified only as total.

The finding of this work that okra vegetable, and especially its seeds, is rich in phenolic compounds with important biological properties like quercetin derivatives, catechin oligomers and hydroxycinnamic derivatives and by also taking in consideration the high amount of glycol-protein and other dietary elements presented, enhance this vegetable nutritional value in human diet (Havsteen, 2002; Le Marchand, 2002; Manach, Williamson, Morand, Scalbert, & Remesy, 2005). In the view of these new data concerning the okra polyphenolic profile, the biological activities and nutritional properties associated to okra chemical composition probably have to be revaluated.

Moreover, the use of a 1.8 μm C18 column gave the possibility to perform a fast separation, with good resolution and high throughput analysis (one analysis every 35 min) compared to conventional columns where the double time usually needed (Innocenti et al., 2007; Romani, Pinelli, Galardi, Mulinacci, & Tattini, 2002; Romani et al., 2006). Also, the low flow rate used (0.2 mL/min) made it possible to decrease solvent consumption and facilitated the connection to the triple quadruple system, compared to conventional column flow rates with 1.5–0.8 mL/min, where a split system is almost always necessary.

4. Conclusions

In conclusion this work was focused in the elucidation of okra polyphenolic profile, an issue with limited knowledge. The qualitative and quantitative analysis demonstrated that this vegetable is rich in polyphenolic compounds, with the seeds to be mainly composed by quercetin derivatives and catechins and skins by quercetin and hydroxycinnamic acid derivatives. Further studies in the structural elucidation of the hydroxycinnamic acid derivatives, the catechins and the other unknown compounds not fully characterized in this work are needed to provide more information about this, for human nutrition, important vegetable.

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