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Effects of Enrichment of Refined Olive Oil with Phenolic Compounds from Olive Leaves

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The possibility of preparing olive oil, with the same nutritional value and stability characteristics found in virgin olive oil, by the enrichment of refined olive oil with olive leaf polyphenols was studied. To obtain antioxidant phenols similar to those found in virgin olive oil, these components were extracted from the leaves of several olive cultivars from the Northern region of Portugal, namely, Carrasca, Ripa, Negruche, Cordovil, Verdeal, Madural, and Bical cultivars, under several conditions. The concentration of a leaf extract required for addition to refined olive oil to obtain the same stability as virgin olive oil was determined. The extract from 1 kg of leaves was sufficient to fortify 50−320 L of refined olive oil to a similar stability as a virgin olive oil sample depending on the metal concentration of the oil, cultivar, and time of the year when the leaves were picked.

KEYWORDS: Olive oil; polyphenols; olive leaves; refined olive oil

INTRODUCTION

Lipid oxidation in virgin olive oil (VOO) is of great concern to the consumer because it not only causes changes in the quality attributes of food, such as shelf life, appearance, and flavor, but also causes a strong decrease in the nutritional value and safety caused by the loss of antioxidants and formation of hydroperoxides and carbonyl compounds. Many epidemiological studies have concluded that the incidence of coronary heart disease (CHD) and certain cancers in the Mediterranean countries is low, suggesting that this is largely due to the relatively safe and even protective diet of this southern area, where VOO is the principal source of fat (*1*). The cause of the potentially beneficial health effect of olive oil has been assigned to both an adequate fatty acid profile and the presence of phenolic compounds, which act as antioxidants. Therefore, the formulation of an antioxidant/atherosclerosis hypothesis led to experimental studies on the possible role of olive oil phenols in the protection against CHD. Animal and in vitro studies suggest that the relatively high concentration of phenolic compounds in VOO may contribute to the healthy nature of this oil (*2*−*7*).

By definition, VOO is consumed unrefined, but a great proportion of the olive oil produced has to be refined to render it edible. In Portugal, the market for extra VOO and virgin oil is limited to 25% of the total olive oil market, with the remaining 75% sold as a mixture of VOO and refined olive oil (ROO) (data from ACASCA, Portuguese Ministry of Agriculture). These olive oils have a much lower content of polyphenols since these compounds are among the substances eliminated during the refining process (*8*). Because no additives can be added to olive oil, since they are considered foreign compounds, ROO needs to be added to VOO to be consumed, diluting the phenolic fraction in the VOO but increasing the content of these compounds in ROO.

Therefore, the total worldwide consumption of olive oil and the limited VOO production capacity indicate the potential for increasing the range of oils offered to the consumer. The possibility of developing higher quantities of olive oil, with the same nutritional value and stability characteristics found in VOO, by the enrichment of ROO with olive leaf polyphenols has been studied.

MATERIALS AND METHODS

Samples of ROO (ROO1 and ROO2) and a sample of VOO were supplied by a local company (CIDACEL, SA). Analytical characteristics of these oils are shown in **Table 1**. Leaf polyphenolic extracts were obtained from Carrasca, Ripa, Negruche, Cordovil, Verdeal, Madural, and Bical cultivars and analyzed by high-performance liquid chromatography (HPLC).

HPLC. The HPLC system comprised a Merck Hitachi chromatograph with a Merck Hitachi L-6200 Intelligent Pump and a 250 mm × 4.6 mm Waters Spherisorb ODS2 5 μm column (Supelco Inc.), coupled to a Merck Hitachi L-4200 UV−vis detector. Components were detected at 280 nm with elution at room temperature. The flow

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Table 1. Analytical Parameters of Olive Oils Tested[a]

	ROO1	ROO2	VOO
acidity (%)	0.4	0.4	0.3
peroxide value (meq/kg)	3	5	4
wax content (mg/kg)	335	265	168
β-sitosterol (%)	93.2	94.7	94.6
steroids (mg/kg)	1424	1587	1724
18:1T (%)	0.09	0.05	0.04
18:2T + 18:3T (%)	0.05	0.03	0.01
16:0 (%)	12.6	12.9	11.2
18:0 (%)	2.3	2.2	3.4
18:1 (%)	75.1	74.4	75.7
18:2 (%)	7.7	7.9	7.9
18:3 (%)	0.7	0.7	0.6

[a] 18:1T = *trans*-octadecenoic acid.

rate was 1 mL min^{-1}; the mobile phase that was used was a mixture of 2% acetic acid in water (pH 3.1) (A) and methanol (B) with a total analysis time of 70 min, and the gradient was as follows: 95% A/5% B for 15 min, changing to 80% A/20% B in 15 min, 70% A/30% B in 10 min, held at 70% A/30% B for 5 min, changing to 50% A/50% B in 5 min, 40% A/60% B in 5 min, 30% A/70% B in 5 min, held at 30% A/70% B for 5 min, and changing to 100% B in 1 min and maintained until the end of the analysis. Samples were analyzed in duplicate using 20 μL of each solution dissolved in methanol. Solvents were HPLC grade. Peaks were identified and quantified by comparison of retention times and areas using a standard solution containing several polyphenols at known concentration, and syringic acid was used as an internal standard.

Isolation of Polyphenols from Leaves. Leaves from each cultivar (50 g) ware macerated in 250 mL of ethanol (or methanol) for 5 days in the dark at room temperature. The extract was separated by filtration, and the solvent was evaporated under vacuum. The residue was taken up in 50 mL of acetone/water (1:1) (v/v). Internal standard (syringic acid) was added except in the extract obtained for the fortification. The aqueous mixture was successively extracted with *n*-hexane and ethyl acetate. Each organic solution was washed with water, the solvent was evaporated, and the extracts were dissolved in ethanol in volumetric flasks (25 mL). The ethanolic extract was then used for the enrichment of ROOs (Carrasca extract) or diluted five times for analysis by HPLC and thin-layer chromatography. Extraction for each sample was performed in duplicate. SPSS 14.0 software was used for statistical analysis by one-way analysis of variance (ANOVA) with the level of significance set at $P < 0.05$.

Isolation of Phenolic Compounds from Olive Oils. Mixtures of phenolic compounds were obtained from VOOs and ROOs by solid-phase extraction (SPE) extraction following the procedure described by Mateos et al. (*9*). Extraction of each sample was performed in duplicate. SPSS 14.0 software was used for statistical analysis by one-way ANOVA with the level of significance set at $P < 0.05$.

Reference Compounds. Hydroxytyrosol was synthesized from 3,4-dihydroxyphenylacetic acid (Sigma-Aldrich Quimica-S.A., Madrid, Spain) according to the procedure of Baraldi et al. (*10*). Oleuropein was purchased from Extrasynthese (Genay, France) or extracted from olive leaves according to the procedure of Gariboldi et al. (*11*). Tyrosol was purchased from Sigma-Aldrich Quimica-S.A. The oleuropein aglycon 3,4-DHPEA-EA was obtained from oleuropein by enzymatic reaction using β-glycosidase (Fluka, Buchs, Switzerland) according to the procedure of Limirioli et al. (*12*). The dialdehydic form of elenolic acid linked to hydroxytyrosol (3,4-DHPEA-EDA) was obtained from olive leaves according to the procedure of Paiva-Martins and Gordon (*13*). Hydroxytyrosol acetate was obtained from hydroxytyrosol according to the procedure of Gordon et al. (*14*). Calibration curves were obtained by plotting the peak area of each phenolic compound as a function of standard concentration. The regression coefficients ranged from 0.996 to 0.999 for all analytes. Quantification of the dialdehydic form of elenolic acid linked to tyrosol (4-HPEA-EDA) was performed using the calibration curve of tyrosol.

Stripped Olive Oil (SROO). Olive oil stripped of natural tocopherols and phenols (SROO) was prepared from commercial VOO by washing with 0.5 M NaOH (Merck) solution and passing twice through an aluminum oxide column (Merck). Complete removal of tocopherols was confirmed by HPLC, according to IUPAC Method 2.432.

Table 2. Polyphenol Composition (% w/w) of Leaf Extracts (Carrasca Leaves Picked at the Beginning of September 2005) Obtained by Maceration with Ethanol and Methanol[a]

	solvent	
compound	methanol	ethanol
hydroxytyrosol	1.27	0.54
tyrosol	0.00	0.00
hydroxytyrosol acetate	0.23	0.23
3,4-DHPEA-EDA	21.83	16.56
oleuropein	11.43	15.24
3,4-DHPEA-EA	3.52	9.24
4-HPEA-EDA	2.50	2.50
total (g/kg)	40.78	44.31

[a] Mean of duplicate samples. Range < 3.

Bulk Oil Samples. The ethanolic phenolic extract from Carrasca leaves was added to bulk ROO (20 g) at the required concentration, and the ethanol was evaporated under vacuum at 37 °C. Samples were stored in open 10 cm diameter Petri plates.

Emulsion Samples. The 30% oil-in-water emulsions (33 g) were prepared in 100 mL Erlenmeyer flasks. They were prepared by adding ethanolic phenolic extract at the required concentration to the oil (10 g), with Tween 20 (0.66 g) as an emulsifier dissolved in the required buffer solution (22.3 g). The mixture was sonicated for 10 min in an ice bath. Buffer solutions that were used were acetate 0.05 M buffer, pH 5.5, and 0.05 M 3*N*-morpholinopropanesulfonic acid (MOPS), pH 7.4. Samples were stored in closed (to prevent water evaporation) 100 mL flasks with 6 cm diameter.

Oxidation Experiments. Bulk olive oils and emulsion samples were oxidized in the dark at 60 °C. Each experiment was performed in triplicate. Isolation of oil from emulsions for analysis was by freezing, thawing, and centrifugation. The progress of oxidation was monitored by determination of the conjugated dienes (CD) (AOCS Official Method Ti 1a-64) and *p*-anisidine value (AV) (AOCS Official Method Cd 18-90). Statistical analysis to determine significant differences in antioxidant activity involved plotting CD or AV against time to determine times to certain values and then applying ANOVA one-way with Tukey's HSD multiple comparison to determine differences significant at the 5% level using SPSS 14.0 software.

Metal Analysis. Metal analysis was performed using atomic absorption spectrometry according to IUPAC method 2.631.

Sensorial Analysis. Sensorial analysis was performed by the Official Virgin Olive Oil Panel (seven panelists) of the Instituto Superior de Agronomia (Lisbon, Portugal).

RESULTS AND DISCUSSION

Effect of Solvent on the Phenolic Extract. The use of the less toxic solvent ethanol as the extraction solvent instead of methanol increased the total amount of polyphenols extracted as well as the percentage of 3,4-DHPEA-EA in the extract (**Table 2**). Therefore, this solvent was then used for the extraction procedures.

Effect of Leaf Storage on the Phenolic Extract Composition. The storage of olive leaves of Carrasca, Ripa, Negruche, Cordovil, Verdeal, Madural, and Bical cultivars at 38 °C for 18 h before extraction decreased the percentage of oleuropein in the extracts and increased the concentration of 3,4-DHPEA-EDA, 3,4-DHPEA-EA, and hydroxytyrosol (**Figure 1**), bringing the composition of the leaf extract closer to the composition of olive oil extract. The increase in the percentage of more lipophilic compounds derived from oleuropein is important since this phenolic compound tends to precipitate from bulk oil and also has a relatively low antioxidant activity in many assays

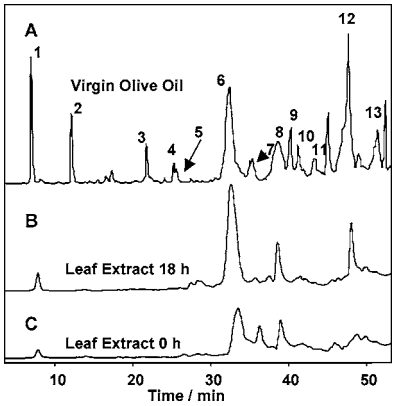

Figure 1. HPLC chromatograms of phenolic extracts isolated from VOO (**A**), Carrasca olive leaf after 18 h in the oven at 38 °C (**B**), and olive leaf without treatment (**C**). Peak identification: 1, hydroxytyrosol; 2, tyrosol; 3, coumaric acid; 4, hydroxytyrosol acetate; 5, tyrosol acetate; 6, 3,4-DHPEA-EDA; 7, oleuropein; 8, 4-HPEA-EDA; 9, pinoresinol; 10, acetoxypinoresinol; 11, lutein; 12, 3,4-DHPEA-EA; and 13, 4-HPEA-EA.

(*14*, *15*). On the other hand, oleuropein derivatives did not show prooxidant effects in previous studies in the presence of common contaminant metals, such as iron and copper (*15*−*17*).

Effect of Cultivar on the Phenolic Extract Composition. All extracts from leaves of different cultivars showed similar relative phenolic compositions after storage at 38 °C for 18 h, but Carrasca and Ripa extracts contained the highest amount of total phenolic compounds (**Table 3**). Nevertheless, the Carrasca cultivar showed a better relative phenolic composition when compared with olive oil phenolic extract. Hydroxytyrosol and secoiridoids were identified in the leaf extracts, but the lignans pinoresinol and acetoxypinoresinol were not detected.

Effect of Extraction Method on the Phenolic Extract. Trituration of stored leaves (Carrasca cultivar) in the extraction solvent gave a reduced yield of extract, when compared with the maceration procedure (**Table 4**), and did not cause major changes in extract composition. Nevertheless, maceration and trituration of leaves slightly increased the yield of extract and decreased the concentration of hydroxytyrosol and tyrosol in the extracts (**Table 4**).

Effect of Phenolic Leaf Extract on the Stability of ROO. The concentration of a leaf extract required for addition to ROO to obtain the same stability as VOO was determined. Bulk ROO samples containing different amounts of phenolic extract were oxidized in the dark at 60 °C, and the progress of oxidation was monitored by determination of the CD and AV. On the basis of the time to CD = 0.4%, it was determined that 185 ± 7 μL of the phenolic extract would need to be added to ROO1 to obtain the same stability as VOO (**Figure 2**). A similar volume (198 ± 6 μL) was calculated from the AV measurements.

This value was much higher than expected. In fact, the three samples containing lower concentrations were not significantly different ($P > 0.05$), and the antioxidant activity of the extract could only be observed for the more concentrated sample (sample 4). The quality of olive oil sample ROO1 was found to be an important factor. Iron was present in this oil at a relatively high concentration (2.3 mg kg^{-1}) (**Table 5**), and olive phenolic components are known to be susceptible to metal-catalyzed decomposition (*17*). Therefore, the stability of the phenolic extract added to ROO1 was studied. After addition of the extract to ROO1, the phenolic compounds were extracted

Table 4. Effect of Extraction Method (Carrasca Leaves Picked at the End of September 2005) on the Extract Polyphenol Composition (g/kg)[a]

	extraction method			
	maceration	trituration	maceration after trituration	trituration after maceration
hydroxytyrosol	0.365	0.065	0.243	0.285
tyrosol	0.330	0.028	0.303	0.260
hydroxytyrosol acetate	0.393	0.250	0.488	0.475
3,4-DHPEA-EDA	16.45	8.28	17.32	16.78
oleuropein	16.90	17.48	25.18	21.58
3,4-DHPEA-EA	3.20	4.40	3.62	3.82
total	37.64 a	30.49 b	47.16 c	43.20 d

[a] Mean of duplicate samples. Range < 3. Different letters within a line indicate samples that were significantly different ($p < 0.05$).

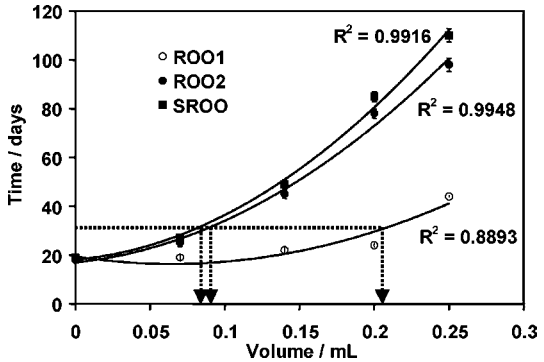

Figure 2. Time (days) for ROO samples with different added volumes of Carrasca phenolic extract to reach the CD content of 0.4%. Means (error bars represent standard deviation) of triplicate stored samples. Arrows indicate times for VOO samples and volumes of phenolic extract with equivalent stability. Time for VOO samples = 32.4 ± 0.9 days.

Table 3. Effect of Olive Leaf Cultivar on the Polyphenol Composition[a]

	polyphenol composition of extracts (%)							
	VOO	Carrasca	Ripa	Madural	Cordovil	Verdial	Bical	Negrucha
hydroxytyrosol	3.56	2.98	0.98	1.44	1.47	1.48	1.50	0.97
tyrosol	1.82	0.89	0.00	0.86	1.76	0.89	1.35	0.00
hydroxytyrosol acetate	0.98	0.48	0.47	0.46	0.94	0.00	0.96	0.93
3,4-DHPEA-EDA	35.0	47.4	42.1	44.9	45.1	42.5	38.3	44.7
oleuropein	5.66	5.52	2.72	21.3	10.9	9.62	8.36	9.49
3,4-DHPEA-EA	42.1	29.9	30.6	8.56	19.8	25.4	31.3	26.1
4-HPEA-EDA	10.9	12.7	23.1	22.5	20.0	20.1	18.2	17.7
total (g/kg)		38.1 a	36.2 ab	36.0 b	14.9 c	27.2 d	21.7 e	23.5 e

[a] Polyphenol composition (% w/w) of leaf extracts after treatment at 38 °C for 18 h (leaves picked in January 2005). Mean of duplicate samples. Range < 3. Different letters within a line indicate samples that were significantly different ($p < 0.05$).

Table 5. Concentration of Metals in Used Olive Oils Tested

	concentration (mg kg^{-1})		
metal	ROO1	ROO2	SROO
As	<0.025	<0.025	<0.025
Cd	<0.008	<0.008	<0.008
Cu	<0.05	<0.05	<0.05
Pb	<0.020	<0.020	<0.020
Fe	2.3	0.06	0.06

after 2 h of contact. A major loss of all phenolic compounds added was observed in ROO1, with smaller losses in the other oils. As shown in **Table 6**, the quantity of phenolics recovered from the oil was similar to that added only in the SROO sample. Thus, it is clear that iron-catalyzed decomposition of the phenolic components added to ROO1 is responsible for the weak antioxidant effect observed during the storage of this oil with added olive leaf extract.

According to **Figure 2**, extraction of 1 kg of Carrasca olive leaves (September 2005) is required to provide extract to fortify 52 ± 2 L of ROO1 so that it has the same stability as VOO. Nevertheless, more quantity of this oil could probably be fortified (up to 150 L) depending on the cultivar and time of the year when the leaves were picked. However, if the iron content of the oil is low, a larger amount of ROO can be fortified by this extract (110−120 L) as calculated from the data for the SROO (122 ± 4 L) and ROO2 (111 ± 3 L) samples. Samples made with better quality ROO (ROO2) or with SROO, which were both less contaminated with iron, containing the same amount of phenolic extract, were much more stable than ROO1.

In emulsions, the volume of same Carrasca phenolic extract needed to fortify ROO2 and SROO to the same stability as VOO was higher (172 ± 6 and 160 ± 3 μL at pH 7 and 162 ± 2 and 130 ± 3 μL at pH 5, respectively), almost double the volume needed for addition to the bulk oil (82 ± 3 and 91 ± 3 μL, respectively) (**Figure 3**). Nevertheless, the assessment of stability in emulsions is much more difficult due to several factors: physical instability over long periods of storage, introduction of metal ions due to the use of a metal probe in the sample during preparation of the emulsion, variation in oil droplet size, and use of buffers that may have metal-chelating effects.

Phenolic Compounds Remaining in the Oil after Addition. The metal content of the ROO was found to be an important factor. Although all of the quality characteristics were in the range of the accepted values in the European Community (**Table 1**), contamination of oil ROO1 by iron was relatively high (**Table 5**) and this contributed to the destruction of a high proportion of the phenolic extract added (**Table 6**). The quantity destroyed was independent of the quantity added above a certain level of addition for a given ROO (**Figure 4**). Assessing the loss of phenolics by the total extract HPLC peak area, 52% of the extract added was lost. Using individual compound peak areas, the concentration lost was 58% for hydroxytyrosol, 52.6% for DHPEA-EDA, and 50% for oleuropein. The lower value

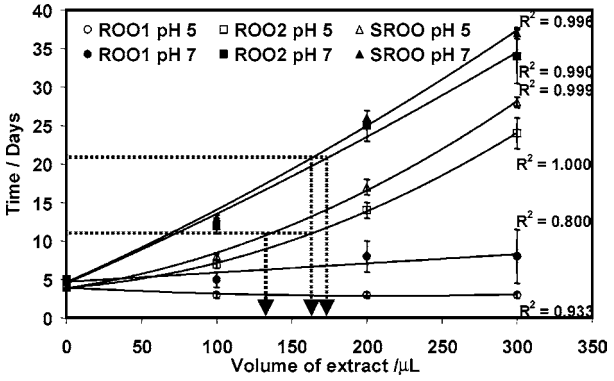

Figure 3. Time (days) for ROO-in-water emulsion samples with different added volumes of phenolic extract to reach the CD content of 0.4%. Means (error bars represent standard deviation) of triplicate stored samples. Arrows indicate times for VOO samples and volumes of phenolic extract equivalent. Time for VOO samples at pH 7 = 21.5 ± 0.6 days. Time for VOO sample at pH 5 = 11.7 ± 0.4 days.

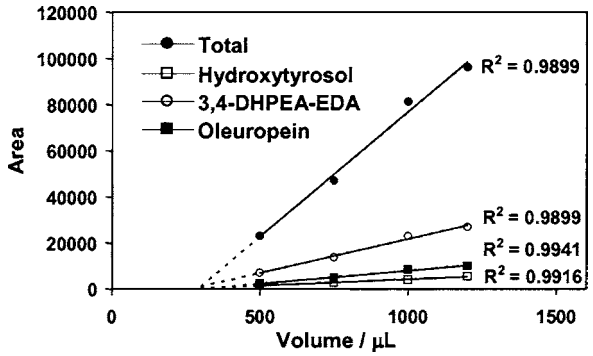

Figure 4. Quantity of phenolic extract that can be extracted from the oil 2 h after addition to ROO assessed by HPLC peak areas for individual compounds or for the total extract.

for oleuropein shows its higher stability in the extract. The loss of phenolic extract in another sample of ROO from a different source was also determined with similar results (data not shown). The loss of phenolic compounds in the ROO sample is of significance since ROO is sold to the consumer mixed with VOO. According to these results, the loss of nutritional quality may occur with this procedure. On the other hand, ROO2 and SROO, both with lower iron concentrations, did not show a significant loss of phenolic compounds after addition.

Effect of Phenolic Leaf Extract on the Taste and Flavor of ROO. Because there is not an official method for ROO sensorial evaluation, the official panel of the Agronomy Institute performed a comparative evaluation. No attributes or defects were observed by the panel for the ROO. The panel did not find significant differences between the flavor of the ROO and the fortified ROO. However, a better score in terms of taste quality was obtained with the fortified ROO.

In conclusion, leaves from the Carrasca cultivar were found to have the highest amount of phenolic compounds of the seven cultivars studied and this cultivar was therefore preferred for

Table 6. Total Area of Phenolic Compound Peaks in the HPLC Chromatograms for Extracts from Olive Oil Samples[a]

	without added extract				plus 500 μL of extract			
	VOO	ROO1	ROO2	SROO	VOO	ROO1	ROO2	SROO
total polyphenol area (×1000)	22.6 ± 0.4	0.1 ± 0.1	0.0 ± 0	0.0 ± 0	69.8 ± 0.5	22.5 ± 0.4	42.2 ± 0.2	45.3 ± 0.3
volume equivalent (μL)					500 ± 1	238 ± 9	447 ± 11	480 ± 12

[a] Extracts were obtained by SPE. Means of duplicate samples ± range.

the extraction procedure. The storage of leaves at 38 °C reduced the total amount of phenols but changed the phenolic composition of the leaf extract so it was closer to the composition of VOO extract. Ethanol, a less toxic solvent, also improved the yield of phenolic compounds in the extraction. Maceration was found to be the best extraction method, but the yield was improved when trituration was also performed. According to the results, it would be necessary to use the extract from 1 kg of leaves to fortify 50−320 L of ROO depending on the metal concentration of the oil, cultivar, and time of the year when the leaves were picked. Experimental studies with phenolic extracts added to ROO should also take into account the effect of metal concentration on catalyzing the destruction of phenolic components.

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