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High Performance Liquid and Thin-Layer Chromatographic Determination of Phenolic Acids in Palm (*Phoenix dactilifera*) Products

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ABSTRACT

Phenolic acids have been determined in the Tunisian currant palm tree products, dates and legmi *(a fermented sap extract). Reversed phase high performance liquid chromatography has been used and results confirmed for the more concentrated compounds by thin-layer chromatography on silica gel.*

Gallic acid, protocatechuic, p*-hydroxybenzoic, syringic, vanillic, caffeic,* p*-coumaric and ferulic acids have been identified and the origin of these compounds is discussed.*

The analysed palm tree products are rich in phenolic acids and are characterized by a high concentration in one particular phenolic acid: ferulic acid for dates, gallic acid for legmi.

INTRODUCTION

Economically and nutritionally, the palm (*Phoenix dactilifera*) is an essential plant in Tunisia. It ensures prosperity for the oases and dates provide a considerable part of the energy in Bedouin foodstuffs. This fruit, in fact, is commonly used for cooking in all of Tunisia. It serves to make sweets, cakes, wedding couscous, etc. Another main palm tree product is *legmi*, a primary

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sap extract collected at dawn. A refreshing morning drink, *legmi* ferments rapidly during the day and turns into an alcoholic beverage by the evening. This beverage is particularly appreciated by Southern and Sahelian populations.

A third product, derived from dates, is also commercialized as 'Thibarine'. It is a matured distilled alcoholic beverage obtained after date fermentation. Thibarine is a coloured liquor, largely consumed within the country, but a small part of the production is also exported.

As with all the other coloured alcoholic beverages (Jouret & Puech, 1975), Thibarine is rich in phenolic acids (Biard *et al.*, 1985).

As well as occurring in higher plants, particularly in the catabolism of aromatic amino acids and flavanoids (Ribereau & Gayon, 1968; Barz & Hösel, 1975; Ebel & Hahlbrock, 1982) phenolic acids are also obtained by hydrolysis of lignin in barrels in which spirits are aged (Jouret & Puech, 1975; Deibner *et al.*, 1976; Puech *et al.*, 1984). They confer a part of the flavour to many food products of vegetable origin, particularly alcoholic drinks (Lichev *et al.*, 1984; Puech *et al.*, 1984).

The purpose of this work is to identify phenolic acids in these products and to discuss their origins.

Reversed phase high performance liquid chromatography (HPLC) was used and the results confirmed for the more concentrated compounds by thin-layer chromatography (TLC) on silica gel. Products were purchased at the local market.

MATERIALS AND METHODS

Apparatus

HPLC: A Waters liquid chromatograph with a μBondapak C_{18} column was used. A model 6000 A delivery system, an U 6 K injector system and a 440 UV absorbance detector was connected. The recorder was an Omniscribe: A (Houston Instruments).

TLC: A chromatographic tank and UV cabinet with a Camag UV lamp de luxe 29000 (254 and 366 nm) was used. The thin-layer system was silica gel with UV indicator (Si 60 F 254, Merk).

Reagents and standard solutions

Solvents and chemical products: dioxane, ether, benzene, methanol, ethanol, sodium sulphate (Na_2SO_4).

Solutions: acetic acid 2%; 2N HCl; sodium carbonate ($NaHCO_3$) in 5% aqueous solution; ferric chloride ($FeCl_3$) in ethanol 1%.

Standard solutions of phenolic acids were prepared using 1 mg per millilitre in dioxane–acetic acid 2% (15:85): *m*- and *p*-hydroxybenzoic, ferulic, syringic, vanillic, gentisic, gallic, protocatechuic, *p*-coumaric, caffeic, benzoic, β- and γ-resorcylic, salicylic and sinapic acids; also, derived reduced compounds: aldehydes (vanillin, *o*-vanillin) and alcohols (vanillic and isovanillic).

Analysed foodstuffs

The products were purchased from local markets and analysed shortly afterwards. Old dates and aged fermented *legmi* were also analysed. Although the external aspects of *legmi* samples were essentially similar (in spite of the well known fact that some extracts are diluted), dates, on the other hand, have different appearances: some, the *deglet nours*, are glazed and pithy, whilst others are firm and brown; old dates are tough and dry.

Analyses were carried out on several samples of *legmi* and dates (*deglet nours*) from an homogeneous lot but estimations were also carried out on samples bought at random and on aged products.

Protocol

Legmi (250 ml) were filtered; dates (300 g) were stoned and crushed in water before being centrifuged at high speed.

Free and combined phenolic acids were extracted from the aqueous phase as previously described (Ben Hassine *et al.*, 1982). First of all, aqueous phases were acidified to pH 2 by 2N HCl and then extracted using ether. The ether phase (A) contains free phenolic acids and the aqueous phase (B) combined phenolic acids. The ether phase (A) was made alkaline using $NaHCO_3$ solution (5%), then acidified (pH = 2) by 2N HCl and extracted once again with ether. Sodium sulphate was added to this ether phase to dry it. After filtration, the ether phase was evaporated (40°C, a rotary evaporator). The dry residue was kept at low temperature and dissolved just before use in eluant for HPLC and TLC.

The combined phenolic acids were released by a smooth hydrolysis of the aqueous phase (B) for 1 h. After cooling, the aqueous phase was extracted with ether. This ether phase (C) was treated as phase (A).

Dry weight of dates was determined by azeotropic water extraction and by water loss after drying (*Pharmacopée Française*, 1983).

The extraction rate was ascertained and calibration curves were set up with internal standard solutions of identified phenolic compounds, according to Yost *et al.* (1980).

HPLC and TLC characteristics

HPLC: Eluent (adapted from Ben Hassine *et al.* (1982)): dioxane–acetic acid 2% (15:85) degassed and filtered through a Millipore membrane under vacuum before use.
Mobile phase flow rate: 1 ml/min
Pressure: 900 psi
Injection: 1 cm/min
Chart speed: 1 cm/min
Internal standard solution: caffeic acid
UV detection: 280 nm
TLC: eluent (from Randerath (1971)).
System 1: benzene, dioxane–acetic acid (90:25:4)
System 2: benzene, methanol acetic acid (45:8:3)
Visualisation: UV and $FeCl_3$ 1%

RESULTS AND DISCUSSION

The migration in TLC of the investigated compounds and the retention times (relative to caffeic acid) in HPLC are shown in Tables 1 and 2.

Polar components eluted typically before non-polar components in the reversed system: gallic acid, with the majority of benzoic acids, appeared first and cinnamic acids were eluted last. It was apparent that the elution rate of phenolic acids increased with the degree of hydroxylation. For an equal degree of hydroxylation, benzoic acids eluted before cinnamic acids. However, salicylic and *o*-coumaric acids, as mentioned by Wolf & Nagel (1976) showed special behaviour. (See Fig. 1).

The detection limit of injected quantities is 0·01 μg and extraction rates are shown in Table 2.

The extraction performs differently for the investigated compounds and there are some difficulties in obtaining an acceptable total extraction of the phenolic acids. Most derived reduced compounds such as aldehydes (vanillin, *o*-vanillin) or alcohols (vanillic, isovanillic alcohol) are not extracted except for vanillin (which is feebly extracted).

Given the detection limit indicated above, it is necessary to take into account that the uncertainty in estimation of the feebly extracted phenol will be greater. It will be more uncertain for *legmi*, which is rich in gallic acid, than for dates.

All phenolic acids of the dates are in both free and combined forms (Table 3). Ferulic acid is the most abundant in the free form. *p*-Coumaric acid, vanillic acid, *p*-hydroxybenzoic acid, protocatechuic acid and syringic acid

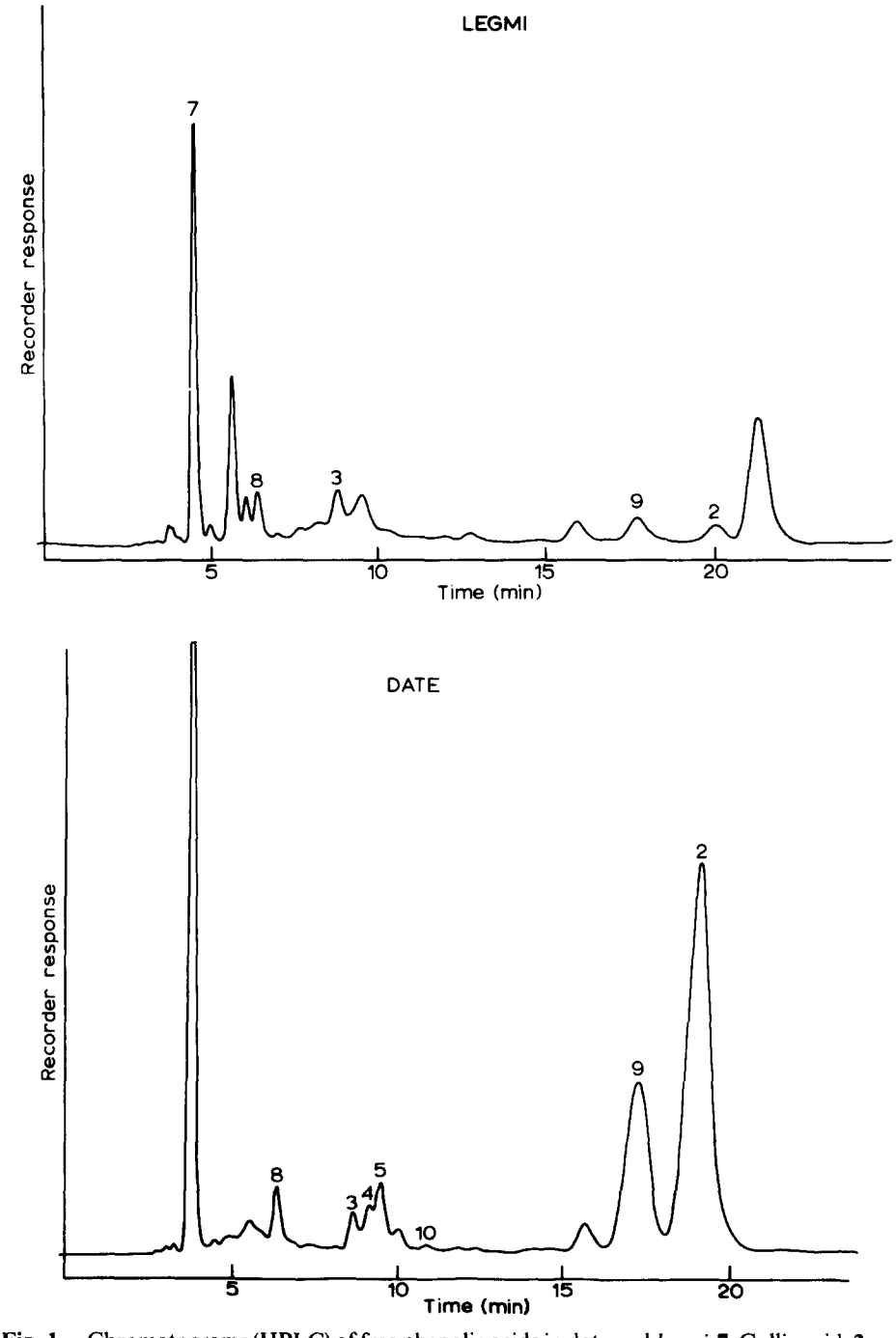


Fig. 1. Chromatograms (HPLC) of free phenolic acids in date and *legmi*. **7**, Gallic acid. **3**, *p*-Hydroxybenzoic acid. **5**, Vanillic acid, **9**, *p*-Coumaric acid. **8**. Protocatechuic acid. **4**, Syringic acid. **10**, Caffeic acid. **2**, Ferulic acid.

TABLE 1
Chromatographic (TLC) Behaviour of Phenolic Compounds: R_f and Colour with $FeCl_3$

Phenolic acids	R_f[a]		*Colour with* $FeCl_3$
	System I	*System II*	
m-Hydroxybenzoic	0·56	0·55	Pale yellow
Ferulic	0·62	0·60	Brown
p-Hydroxybenzoic	0·55	0·51	Yellow
Syringic	0·48	0·55	Brown
Vanillic	0·54	0·53	Pale brown
Gentisic	0·32	0·28	Violet
Gallic	0·11	0·13	Blue–violet
Protocatechuic	0·28	0·26	Blue–violet
p-Coumaric	0·52	0·51	Red–orange
Caffeic	0·26	0·33	Blue–green
β-Resorcylic	0·50	0·46	Brown
γ-Resorcylic	0·49	0·52	Pale yellow
Salicylic	0·63	0·62	Red
Sinapic	0·57	0·62	Pale violet
Benzoic	0·80	0·72	Yellow

System I: benzene–dioxane–acetic acid (90:25:4).
System II: benzene–methanol–acetic acid (45:8:3).
[a] Temperature variations may interfere with the mentioned value.

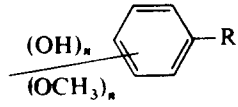
have also been identified, the latter being partly released by the hydrolysis. Gallic acid has not been identified in dates but its extraction rate is low and consequently it may not have been possible to detect it.

On the other hand, this phenolic acid is the most abundant in *legmi* (Table 4). Protocatechuic acid is present in smaller quantities. The *p*-hydroxybenzoic, *p*-coumaric and ferulic acids exist only in the free form, whereas syringic acid exist as a combined form. The high concentration of gallic acid, especially as the free form, has been established elsewhere in wine just after fermentation (Dadic *et al.*, 1980). This is consistent with the fact that *legmi* is a fermented product and can be considered as a sort of palm tree wine after ageing.

Quantitative estimation of phenolic acids shows non-negligible variations from one sample to another with products bought at random from the local market. It was also noticed that ageing causes a decrease in the phenolic acid concentrations of the samples.

The large diversity of commercialized species may interfere; also, the time between the crop collection and sale is variable and the degree of maturity of the fruit at harvest can be different. Then, obviously, *legmi* is an artisanal

TABLE 2
HPLC Behaviour of Phenolic Compounds: Relative Retention Time (RRT versus caffeic acid) and Extraction Rate



Products	$(OH)_n$ $n=$	$(OCH_3)_n$ $n=$	*RTT versus caffeic acid*	*Extraction rate* (%)
Benzoic acids R = (COOH)				
Benzoic	0	0	1·723	—
Salicyclic	1	0	2·102	—
m-Hydroxybenzoic	1	0	0·901	84·6
p-Hydroxybenzoic	1	0	0·760	83·6
Protocatechuic	1	0	0·578	54·6
α-Resorcylic	2	0	0·536	51·3
β-Resorcyclic	2	0	1·010	81·6
γ-Resorcylic	2	0	0·619	61·1
Gentisic	2	0	0·739	72·7
Gallic	3	0	0·432	7·8
Vanillic	1	1	0·828	85·7
Syringic	1	2	0·796	52·8
Cinnamic acids R = (CH=CH—COOH)				
o-Coumaric	1	0	2·364	—
m-Coumaric	1	0	1·796	—
p-Coumaric	1	0	1·437	79·7
Caffeic	2	0	1·000	58·2
Ferulic	1	1	1·567	67·3
Sinapic	1	2	1·455	—
Aldehydes R = (CHO)				
Vanillin	1	1	0·875	22·2
o-Vanillin	1	1	1·255	2·7
Alcohols R = (CH_2OH)				
Vanillic	1	1	0·473	0
Isovanillic	1	1	0·512	0

product and is characterized by heterogeneity of production conditions. We have noticed, for instance, that sugar and water are often added to the *legmi* sold in Monastir.

Another argument may be considered. The phenolic acids are intermediate compounds of metabolism in higher plants and are linked by numerous catabolic or anabolic connections in plant metabolic pathways. They are a part of the dynamic role of polyphenols in higher plants (Ribereau-Gayon,

TABLE 3
Evaluation of Phenolic Acids in Dates

Phenolic acids concentration (μg/kg dry matter)	*Proto-catechuic*	*p-Hydroxy-benzoic*	*Syringic*	*Vanillic*	*Caffeic*	*p-Coumaric*	*Ferulic*	*Total*
Homogeneous samples ($n = 5$)								
Free:								
Concentration	186	77·3	461	539	55·1	434	3 252	5 005
%	3·7	1·5	9·2	10·8	1·1	8·67	65·0	100
$\bar{\sigma}$	0·71	1·51	3·17	1·86	1·98	1·39	4·40	5·11
Combined:								
Concentration	97·9	47·9	260	40·0	4·2	84	326	859
%	11·4	5·6	30·2	4·6	0·5	9·8	37·9	100
$\bar{\sigma}$	3·73	1·84	4·53	3·42	1·81	2·25	2·49	11·30
Total:								
Concentration	284	125	721	579	59·1	519	3 578	5 864
%	4·8	2·1	12·3	9·8	1·0	8·8	61·0	100
$\bar{\sigma}$	3·53	2·58	3·02	3·32	3·14	2·13	4·91	7·18
Old samples								
Free	38·2	45·9	238	73·6	ND	93·1	927	1416
Combined	20·7	ND	150	18·0	ND	102	104	396
Samples at random								
No. 1—Free	137	75·9	310	131	66·8	207	2 700	3 628
Combined	163	24·9	372	50·9	ND	109	306	1 025
No. 2—Free	122	154	489	160	112	493	1 557	3 085
Combined	121	121	397	73·9	83·7	50·12	240	1 023
No. 3—Free	159	134	599	170	116	538	2700	4 417
Combined	65·0	106	119	33·5	6·21	84·2	472	886

ND, Not determined

TABLE 4
Evaluation of Phenolic Acids in *Legmi*

Phenolic acids concentration (μg/litre)	*Gallic*	*Proto-catechuic*	*p-Hydroxy-benzoic*	*Syringic*	*p-Coumaric*	*Ferulic*	*Total*
Homogeneous samples (n = 5)							
Free:							
Concentration	1 725	62·1	41	—	23·5	5·57	1 858
%	92·9	3·2	2·2	—	1·3	0·3	100
$\bar{\sigma}$	41·8	1·24	1·19	—	0·65	1·67	41·9
Combined:							
Concentration	184	13·8	—	66·2	—	—	264
%	69·6	5·2	—	25·1	—	—	100
$\bar{\sigma}$	2·99	0·98	—	11·4	—	—	9·71
Total:							
Concentration	1 934	75·7	41·1	66·3	23·5	5·57	2 124
%	91·0	3·6	1·9	3·1	1·1	0·3	100
$\bar{\sigma}$	46·2	1·82	1·19	11·4	0·65	1·67	51·2
Old samples							
Free	408	40·8	29·2	—	19·5	72·2	570
Combined	79·7	20·4	—	119	—	—	219
Samples at random							
No. 1—Free	2 892	117	47·1	—	36·9	106	3 199
Combined	986	34·0	—	153	—	—	1 173
No. 2—Free	5 189	69·8	36·5	—	29·4	ND	5 325
Combined	606	50·3	—	499	—	—	1 156
No. 3—Free	3 837	74·2	38·3	—	31·6	ND	3 981
Combined	2 099	37·8	—	ND	—	—	2 137

ND, Not determined

1968; Barz & Hösel, 1975; Ebel & Hahlbrock, 1982). Thus, *p*-coumaric, caffeic, protocatechuic, ferulic, *p*-hydroxybenzoic and vanillic acids come from aromatic amino acid catabolism. Some phenolic acids are also combined with glucosides: *p*-coumaric, caffeic and ferulic acids may be found as heteroside complexes. Other complex associations exist in the plant. Dactilifric acid (3-*o*-caffeoyl-shikimic acid) has been identified in green fresh dates (Maier *et al.*, 1964). Hydrolysis releases caffeic and shikimic acid. Shikimic acid can itself change, by several metabolic pathways, into aromatic amino acids (phenolic acid precursors) and into gallic and protocatechuic acids (Ribereau-Gayon, 1968).

It is thus not surprising, under these conditions, to obtain variable concentrations of phenolic acids in vegetable products, particularly when the other variables described, such as maturity, conservation, etc., are considered, and it will be interesting to define these further.

However, despite the diversity of phenolic acids identified in palm tree products, it appears that these products are characterized by a notably high concentration of one particular phenolic acid: ferulic acid for dates and gallic acid for *legmi*. Investigation of their role would be interesting.

This high concentration of particular phenolic acids is not observed in manufactured products like Thibarine. In fact, Thibarine has all the phenolic acids identified in the date (Biard *et al.*, 1985) but some do not originate in the fruit but from hydrolysis of the lignin which initially releases aromatic aldehydes such as syringaldehydes, vanillin, coniferaldehyde and sinalpaldehyde; these aldehydes then being oxidized into syringic, *p*-hydroxybenzoic, ferulic and vanillic acids.

In consequence, determination of whether a high concentration of a particular phenolic acid is characteristic of one plant product and definition of the role it plays, appear to be worthy of interest.

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