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Detailed Polyphenol and Tannin Composition and Its Variability in Tunisian Dates (*Phoenix dactylifera* L.) at Different Maturity Stages

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Ⓢ *Supporting Information*

ABSTRACT: The polyphenol profile of two Tunisian varieties of dates including flavanols, flavonols, flavones, and hydroxycinnamates was characterized. Three tissue zones (flesh, peel, and stone) and three maturity stages were considered. Phenolic compounds were analyzed using reversed phase high-performance liquid chromatography coupled to UV−visible and electrospray mass spectrometry. Procyanidin oligomers and polymers were characterized and quantified using phloroglucinolysis prior to HPLC analysis. Procyanidin polymers based on (−)-epicatechin structure were by far the most concentrated polyphenols in ripe dates, accounting for 95% of total polyphenols with an average concentration of 14 g/kg in the fresh edible parts of the fruit. Interestingly, procyanidins were also highly concentrated in the stones. The concentration and average degree of polymerization (DP*n*) of the procyanidins decreased according to maturity. Other phenolics, including caffeoylshikimic acid hexoside, caffeoyl-sinapoyl monohexoside and dihexoside, and acetylated flavonols, were tentatively identified for the first time in the fruit.

KEYWORDS: *dates, procyanidins, phloroglucinolysis, condensed tannins, LC-MS*

INTRODUCTION

The date palm is a very widespread fruit tree in most Arabian countries.[1] Before the discovery of oil, together with camel milk and fish, the date was the main source of food in the gulf region, the United Arab Emirates (UAE), and North Africa in particular. The date is an important crop in arid and semiarid regions throughout the world and still plays an essential role in the diet of local inhabitants.[2] Furthermore, a significant proportion of date production is exported to European (EU) countries. Although Tunisia accounts for only 2% of the world's date production, its share in global exports is 21% and it represents 55% of EU imports in value.[3]

It is now admitted that high fruit and vegetable consumption is associated with a reduced risk of several chronic diseases such as cancer, cardiovascular disease, coronary heart disease, and atherosclerosis, as well as neurodegenerative diseases and inflammation. Moreover, more evidence is now available indicating that polyphenols could be involved, at least partially, in some of those effects.[4] From this point of view, dates can be considered as an interesting source of polyphenols in the diet. In addition, a significant part of the date production is processed into derivative products including jam, frosting, juice, and syrup.[5] Consequently, a large quantity of date stones and nonmarketed fruits could be used as a waste material that could be used on the basis of its high level of bioactive phenolics and dietary fiber.[1,6]

Dates are considered as climacteric fruits having a low ethylene production rate.[7] They go through five stages of development known by their Arabic names: *Hababouk*, *Kimri*, *Khalal*, *Rutab*, and *Tamar*.[8] Many studies have discussed the physical and chemical development of dates as they go through these stages.[1,7] In the Khalal stage weight gain is slow, the sucrose starts to be converted to glucose and fructose, the moisture content decreases, and the fruits lose their astringency. In some varieties, the latter process occurs rapidly, thus making the fruit palatable at the Khalal stage. The tips of the fruit start turning brown as they enter the Rutab stage of ripening, which is characterized by a decrease in weight due to moisture loss and the conversion of sucrose into invert sugar (the degree depends on the variety), as well as browning of the skin and softening of the tissues.[1] In some varieties, the latter process occurs early, making the fruit palatable at the Kimri stage.

The total phenolic content in dates is usually estimated using the colorimetric Folin−Ciocalteu method and varies greatly according to variety.[9] The use of different phenolic standards as well as differences in the units used to express the data (fresh or dried matter) render the quantitative comparison of published data difficult. For instance, Al-Farsi et al.[9] mentioned an average phenolic content ranging from 134 to 280 mg of ferulic acid equivalents (FAE) per 100 g of fresh weight dates, whereas it ranged from only 2.5 to 8.4 mg of gallic acid equivalents (GAE) per 100 g of fresh fruit according to Mansouri et al.[10]

Phenolic compounds in dates are mainly hydroxycinnamates (HCA) and flavonoids (Figure 1). Caffeoylshikimic acids are characteristic phenolic compounds found in the *Palmae* family.[11] In dates, 5-*O*-caffeoylshikimic acid is widespread, although other isomeric forms are found in some varieties.[10]

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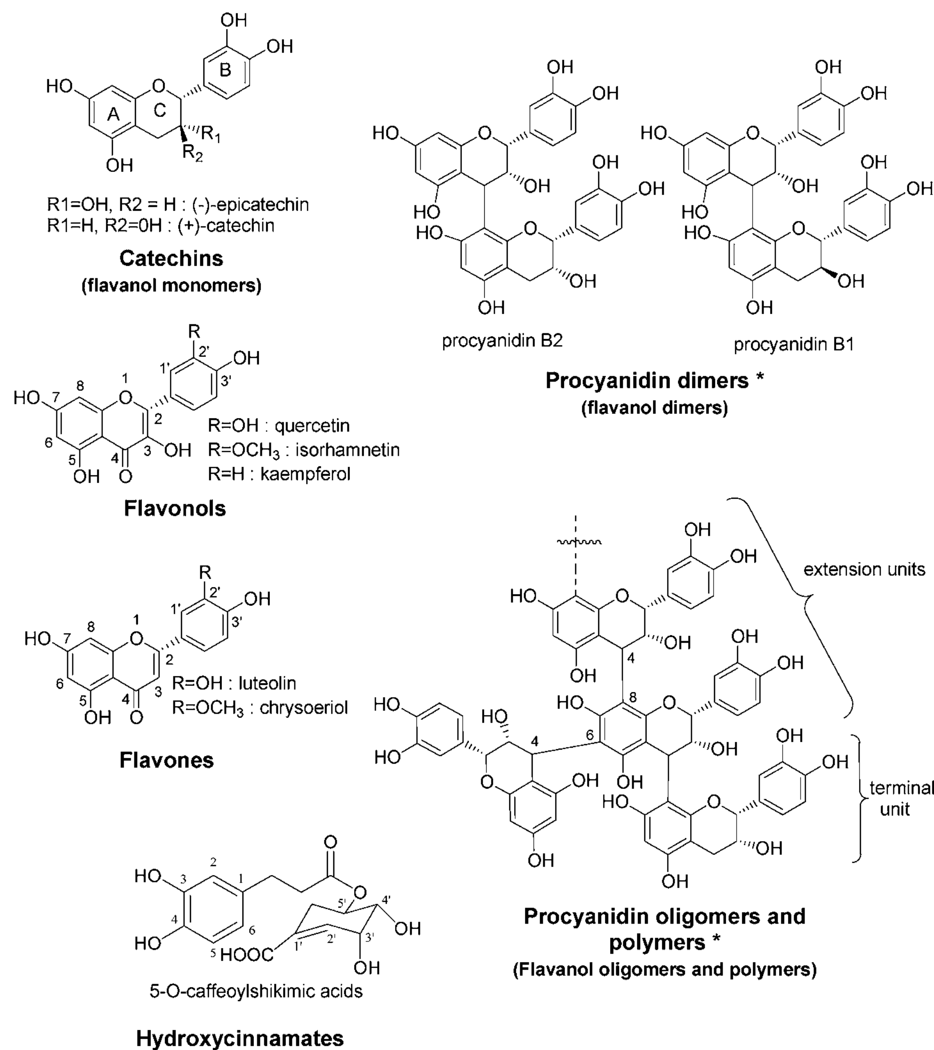

Figure 1. Chemical structures of phenolic compound classes in dates. * Procyanidin dimers, oligomers, and polymers correspond to "condensed tannins".

According to the literature,[10,12] date flavonoids are mainly flavones, flavonols (FO), and flavanols (FAs), the latter including catechin monomers, procyanidin (PC) oligomers, and PC polymers (i.e., condensed tannins) (Figure 1). PC oligomers, up to decamers, have been characterized in dates using liquid chromatography−electrospray ionization−tandem mass spectrometry (LC-ESI/MS/MS) in positive ion mode.[12] The FO and flavone conjugates identified were mainly luteolin and quercetin glycosides and sulfates, of which some were methylated.[12]

However, only a few published works provide detailed data concerning the quantification of the individual classes of phenolic compounds in dates. According to previous publications, only the phenolic acids in date stones have been quantified using HPLC.[13]

Although major organoleptic changes (i.e., loss of astringency and browning) are likely related to major changes in the phenolic composition of dates, it is important to emphasize the lack of information concerning the qualitative and quantitative aspects of individual phenolic compounds during the maturation of dates. The aim of this study was therefore to identify and quantify phenolic compounds in three tissue zones of fruits and evaluate changes during maturation. Some individual phenolic molecules in the date samples were characterized by comparison with available standards and interpretation of the LC-ESI-MS and MS/MS data in negative mode and UV−visible data corresponding to the main chromatographic peaks.

Particular attention was paid to the PC class, which was characterized and quantified using two different approaches: (i) direct analysis of well HPLC-separated individual procyanidin oligomers in fruit powder methanol extracts; (ii) the use of an acidic cleavage method in the presence of an excess of phloroglucinol (i.e., phloroglucinolysis).[14] Phloroglucinolysis was directly applied to fruit powders without prior solvent extraction followed by HPLC analysis of the reaction medium. This enabled the total PC concentration including both extractable and nonextractable procyanidins to be determined. In addition, the method enabled average degree of polymerization (DP*n*) and nature and proportion of the constitutive FA units to be determined.[14,15]

■ MATERIALS AND METHODS

Solvents, Reagents, and Phenolic Standards. Formic acid, ascorbic acid, phloroglucinol, hydrochloric acid, and sodium acetate were purchased from Merck (Darmstadt, Germany). HPLC gradient grade quality acetonitrile and methanol were purchased from Biosolve Ltd. (Valkenswaard, The Netherlands). Sodium fluoride was from

Prolabo (Fontenay-sous-bois, France). Glacial acetic acid was purchased from Biosolve Ltd. Ultrapure water was obtained using a Milli-Q water system (Millipore, Bedford, MA, USA).

(−)-Epicatechin (EC), (+)-catechin (CAT), chlorogenic acid, and quercetin were purchased from Sigma (Sigma-Aldrich, Germany). Procyanidins B1 and B2, luteolin, isorhamnetin, kaempferol, chrysoeriol, quercetin 3-*O*-galactoside (hyperoside), quercetin 3-*O*-rutinoside (rutin), quercetin 3-O-glucoside (isoquercitrin), isorhamnetin 3-*O*-rutinoside, kaempferol 3-*O*-rutinoside, and luteolin 3-*O*-glucoside were purchased from Extrasynthese (Lyon, France).

Plant Materials. The fruits of two Tunisian varieties (Deglet Nour and Ftimi) of date (*Phoenix dactylifera* L.) were harvested in 2009 in the regions of Kebili, Tozeur, and Douz. Three maturity stages were considered for polyphenol analysis: Kimri, Khalal, and Tamar (Figure 2A),[8,16] designated stages 1, 2, and 3, respectively, in the present paper. These correspond to physiological stages well described in the literature by Al-Shahib et al.:[16] Briefly, Kimri corresponds to green fruits that have reached their final size; Khalal corresponds to fruits for which the color of the skin has turned red-purple; Tamar corresponds to soft and dark fruits at commercial maturity. The protocol for plant materials preparation was performed in triplicate: Three batches of 10 fruits were randomly constituted for each variety, maturity stage, and geographic origin and were treated according to the method described by Guyot et al.[17] Tissues were separated manually (Figure 2B), and a sodium fluoride solution (1 g L^{-1} in water) was sprayed on the tissues to inhibit oxidation. The tissues were then freeze-dried and transformed into a fine homogeneous powder by crushing with an electrical crusher (Retsch, model YGG, Bioblock Scientific). The powders were kept under vacuum in a desiccator until analysis.

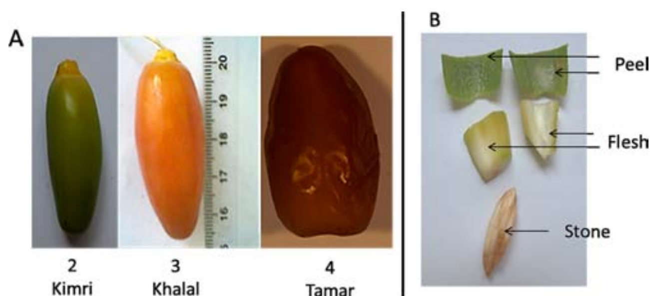

Figure 2. Deglet Nour variety dates: the three maturity stages (A) and three tissue zones (B) considered in the study.

Methanol Extraction of Freeze-Dried Powders. Simple polyphenols, including monomeric catechins, low molecular weight PCs, HCA, and FOs, were extracted from the powders using acidified methanol. Accurately weighed aliquots of powder (10−60 mg) were extracted using 1 mL of pure methanol containing 1% v/v acetic acid for 15 min in an ultrasonic bath (Brasson 2200, USA). The mixture was then filtered on PTFE filters (0.45 μm, Uptidisc Interchim, France). The filtrate was then ready for HPLC analysis.

Acidolysis in the Presence of Phloroglucinol. Phloroglucinolysis leads to the depolymerization of PC structures by converting the FA extension units into its corresponding carbocations and the terminal units into monomeric FAs. Carbocations immediately combine with phloroglucinol, leading to the formation of flavanyl−phloroglucinol adducts.[18] By distinguishing between terminal and extension PC units, the nature and proportion of the PC constitutive units can be determined by HPLC analysis of the reaction media[19] and their average degree of polymerization (DP*n*) calculated. The total PC concentration in the samples can also be determined from the sum of all the units (terminal + extension units).

The phloroglucinolysis method was adapted from Kennedy.[14] Freeze-dried date powders (30 mg) were treated with a 0.3 N HCl solution in MeOH containing 75 g/L phloroglucinol and 10 g/L ascorbic acid at 50 °C for 50 min and then combined with 1.2 mL of aqueous sodium acetate to stop the reaction. The mixture was filtered on PTFE filters (0.45 μm, Uptidisc Interchim). The filtrate was then ready for HPLC analysis.

RP-HPLC of Methanol Extracts and Phloroglucinolysis Reaction Media. The samples were injected into a RP-HPLC system, which included an automatic injector model WISP 717 (Waters, Milford, MA, USA) thermostated at 4 °C, a gradient pump model 600 (Waters), and a diode array detector model 996 (Waters). The column was an RP18 Purospher end-capped 5 μm, 80 Å, 4 × 250 mm (Merck, Darmstadt, Germany) equipped with a 4 × 4 mm guard column of the same RP material and thermostated at 30 °C. The solvent system was a gradient of aqueous acetic acid, 2.5% v/v (solvent A), and acetonitrile (solvent B). The following gradient was applied at a constant flow rate of 1 mL/min: initial, 3% B; 0−5 min, 9% B linear; 5−15 min, 16% B linear; 15−45 min, 50% B, linear followed by washing and reconditioning of the column. The solvents were continuously degassed with helium. The injection volume was 10 μL.

The acquisition, integration, and processing of the signal were controlled using Millennium software 2010 version 2.1. Simultaneous monitoring was performed at 280 nm for quantification of flavan-3-ols, 320 nm for HCA and 350 nm for FOs. Spectra were recorded between 200 and 600 nm. Phenolics were identified on the basis of their retention times and characteristic spectra in comparison with available standards. Quantifications were performed using the calibration curves of selected standards: HCA were quantified at 320 nm as chlorogenic acid equivalents, flavan-3-ols as (−)-epicatechin equivalents at 280 nm, and FOs as quercetin-3-*O*-galactoside (hyperoside) equivalents at 350 nm. With the mean weight of one fruit and the dry matter of the fruits taken into account, the quantitative values were then converted and expressed in milligrams in one fresh fruit.

LC-DAD-ESI-MS and MS/MS for Specific Characterization of Phenolic Compounds. To obtain more detailed information concerning the identification of the phenolic compounds, crude methanol extracts (see paragraph above) of selected methanol extracts were analyzed using HPLC coupled with mass spectrometry. Analyses were performed using an LC-DAD-ESI-MS system composed of an SCM1000 degasification system (ThermoQuest, San Jose, CA, USA), an automatic injection system (ThermoFinnigan, San Jose, CA, USA), a binary pump 1100 series (Agilent Technologies, Palo Alto, CA, USA), and a photodiode array detector Spectra system UV6000LP (ThermoFinnigan). The mass spectrometer was an ion trap LCQ Deca (ThermoFinnigan) equipped with an electrospray ionization source (ESI). The column and the HPLC gradient conditions were the same as described above for analysis of the phloroglucinolysis media apart from the composition of the HPLC solvents: solvent A (aqueous formic acid, 0.1% v/v) and solvent B (acetonitrile containing 0.1% formic acid v/v). The HPLC flow (1 mL/min) was split at the diode array detector outlet to obtain a flow rate of 0.2 mL/min at the ESI source inlet.

The MS spectra were first acquired in full scan negative ionization mode in the m/z 50−2000 range to obtain the signals corresponding to the deprotonated $[M - H]^-$ molecular ions. The source parameters were as follows: spray voltage (5 kV), capillary voltage (12.0 V), sheath gas (67 arbitrary units), auxiliary gas (5 arbitrary units), and capillary temperature (240 °C). Nitrogen was used as the nebulizing gas. Each peak clearly detected on the total ion current chromatograms (TIC) was further investigated by LC-ESI/MS/MS acquisition of the corresponding molecular ion. Helium gas was used as a collision gas, and the potential change defining the collision energy was optimized in the range 25−35% (arbitrary units) to optimize the production of both parent and daughter ions.

Acidic Hydrolysis. Acid hydrolysis was used to confirm the identification of the FO or flavone conjugate aglycones present in the samples.[20] One milliliter of the methanol extract was mixed with 1 mL of 2 N HCl, and the mixture was heated at 90 °C for 30 min in a closed glass vial. The sample was neutralized by the addition of 1 mL of 2 N NaOH before LC-ESI/MS analysis in the same conditions as described above except for the MS/MS collision energy, which was set at 50% (arbitrary units) to obtain adequate fragmentation of the aglycone molecular ions.

Table 1. Identification of Phenolic Compounds in the Peel of the Deglet Nour Variety

peak	identification	stages[a] 1	2	3	RT[b] (min)	λ max (nm)	$[M - H]^-$	major MS/MS product ions
	hydroxycinnamates							
1	caffeoylshikimic acid hexoside	*	*	*	10.3	319	497	497 (47), 341 (45), 335 (100), 179 (14)
2	4-caffeoylshikimic acid	*	*	*	14.4	327	335	291 (20), 179 (100), 161 (96), 135 (24)
3	5-caffeoylshikimic acid	*	*	*	15.0	328	335	317 (6), 291 (22), 179 (100), 161 (25), 135 (13)
4	3-caffeoylshikimic acid	*	nd	nd	16.0	327	335	179 (100), 161 (4), 135 (23)
5	caffeoylshikimic acid hexoside	nd	*	*	12.0	319	497	341 (9), 335 (100), 179 (8)
6	caffeoylsinapoyl hexoside	nd	*	*	17.3	326	547	385 (16), 223 (100), 205 (72)
7	dicaffeoylsinapoyl hexoside	nd	*	*	18.4	327	709	547 (37), 503 (100), 295 (39), 223 (28), 205 (11)
	flavonols and flavones							
8	quercetin rhamnosyl-hexoside sulfate	*	nd	nd	20.7	269, 341	689	609 (29), 387 (30), 301 (100)
9	quercetin 3-*O*-rutinoside (rutin)[c]	*	*	*	21.2	255, 348	609	301 (100), 300 (70), 271 (9)
10	quercetin hexoside sulfate	*	*	*	22.1	256, 349	543	463 (3), 301 (100), 241 (13)
11	quercetin acetyl-hexoside	*	*	*	23.6	276, 333	505	463 (45), 301 (100), 300 (76)
12	isorhamnetin-3-*O*-rutinoside[c]	*	*	*	24.0	276, 337	623	623 (26), 315 (100), 300 (10)
13	isorhamnetin hexoside	*	*	*	25.0	277, 339	477	357 (21), 315 (60), 314 (100) 299 (11)
14	chrysoeriol rhamnosyl-hexoside	*	*	*	25.4	256, 352	607	607 (70), 299 (100), 284 (10)
15	isorhamnetin acetyl-hexoside	*	*	*	27.6	269, 356	519	519 (12), 357 (1), 315 (100), 300 (3)
16	quercetin 3-*O*-glucoside (isoquercitrin)[c]	nd	*	*	23.2	276, 351	463	301 (100), 300 (64)
17	chrysoeriol hexoside sulfate	*	*	*	25.1	256, 354	541	461 (1), 299 (100), 284 (3), 241 (25)
18	chrysoeriol hexoside	nd	*	*	26.6	269, 351	461	461 (100), 299 (17)
	flavan-3-ols							
19	procyanidin B1[c]	*	*	*	11.7	278	577	559 (17), 451 (48), 425 (100), 407 (37), 289 (24)
20	(+)-catechin[c]	*	*	*	13.5	279	289	245 (100), 205 (38), 179(20)
21	procyanidin B2[c]	*	*	*	14.0	278	577	559 (16), 451 (62), 425 (100), 407 (53), 289 (26)
22	procyanidin dimer	*	*	*	15.1	281	577	559 (24), 451 (74), 425 (100), 407 (37), 289 (33)
24	(−)-epicatechin[c]	*	*	*	16.5	279	289	245 (100), 205 (37), 179 (13)
25	procyanidin trimer	*	*	*	18.2	279	865	847 (26), 739 (97), 713 (66), 695 (100), 577 (49)
26	procyanidin tetramer	*	*	*	19.2	281	1153	1027 (64), 1001 (51), 983 (97), 865 (100), 575 (55)
27	procyanidin pentamer	*	*	*	19.9	281	1441	1151 (85), 863 (100), 737 (20), 575 (35)
	procyanidin dimer	nd	*	*	19.4	279	577	559 (27), 451 (76), 425 (100), 407 (60), 289 (33)

[a]Stages of maturity: 1, Kimri; 2, Khalal; 3, Tamar. *, detected; nd, not detected. [b]RT, retention time. [c]Compounds identified against authentic standard.

Statistical Analysis. Because of the significant quantity of data collected corresponding to several variation factors (variety, maturity stage, geographic origin, triplicates of each sample), multiple-way analysis of variance (ANOVA) was chosen to highlight the most influential factors related to polyphenol concentrations in the three tissue zones in question.

A three-way analysis of variance on the polyphenol concentration data was performed using Statgraphics plus Software v. 5 (Manugistics Inc., Rockville, MD, USA). Differences at $P < 0.05$ were considered to be significant.

RESULTS AND DISCUSSION

Identification and/or Structural Characterization of the Phenolic Compounds in Date Tissues. Reversed phase HPLC coupled with UV−visible and MS detection in negative mode was used to detect and characterize the phenolic compounds in the crude methanol extracts prepared from three tissue zones of the fruits of two date varieties harvested at three successive maturity stages. The identification or partial characterization of individual compounds is presented in Table 1. As all phenolic compounds absorb UV light at 280 nm, the UV chromatograms at 280 nm were used as a first step for the detection of individual compounds. The complete UV−visible spectrum of each chromatographic peak then allowed us to classify these compounds into a particular phenolic class (i.e., HCA, FOs, or FAs). Finally, MS and MS/MS data were collected to complete the identification. Compounds were considered as formally identified (FI) when all data (retention time, UV spectrum, and MS/MS spectrum) correctly matched those of the available standard. For the other cases, compounds were tentatively characterized on the basis of their UV−visible, MS, and MS/MS data and by comparison with data in the literature. Tandem mass spectrometry analyses of methanol extracts subjected to acidic hydrolysis were also performed to confirm the nature of some FO and flavone conjugate aglycones.

Hydroxycinnamates. Seven compounds eluted in the 10−19 min zone of the chromatogram (Figure 3a,b), numbered 1−7, and were identified as HCA according to their UV−visible spectra, which were very similar to the spectrum of caffeic acid. Compounds corresponding to peaks 2, 3, and 4 showed deprotonated molecules $[M - H]^-$ at m/z 335. A main product ion at m/z 179 corresponding to caffeic acid was observed on the MS/MS spectra of all three isomers (Table 1). This loss of 156 amu corresponded to the molecular weight of shikimic acid. These compounds were thus identified as the three caffeoylshikimic acid isomers (CSA) that have already been

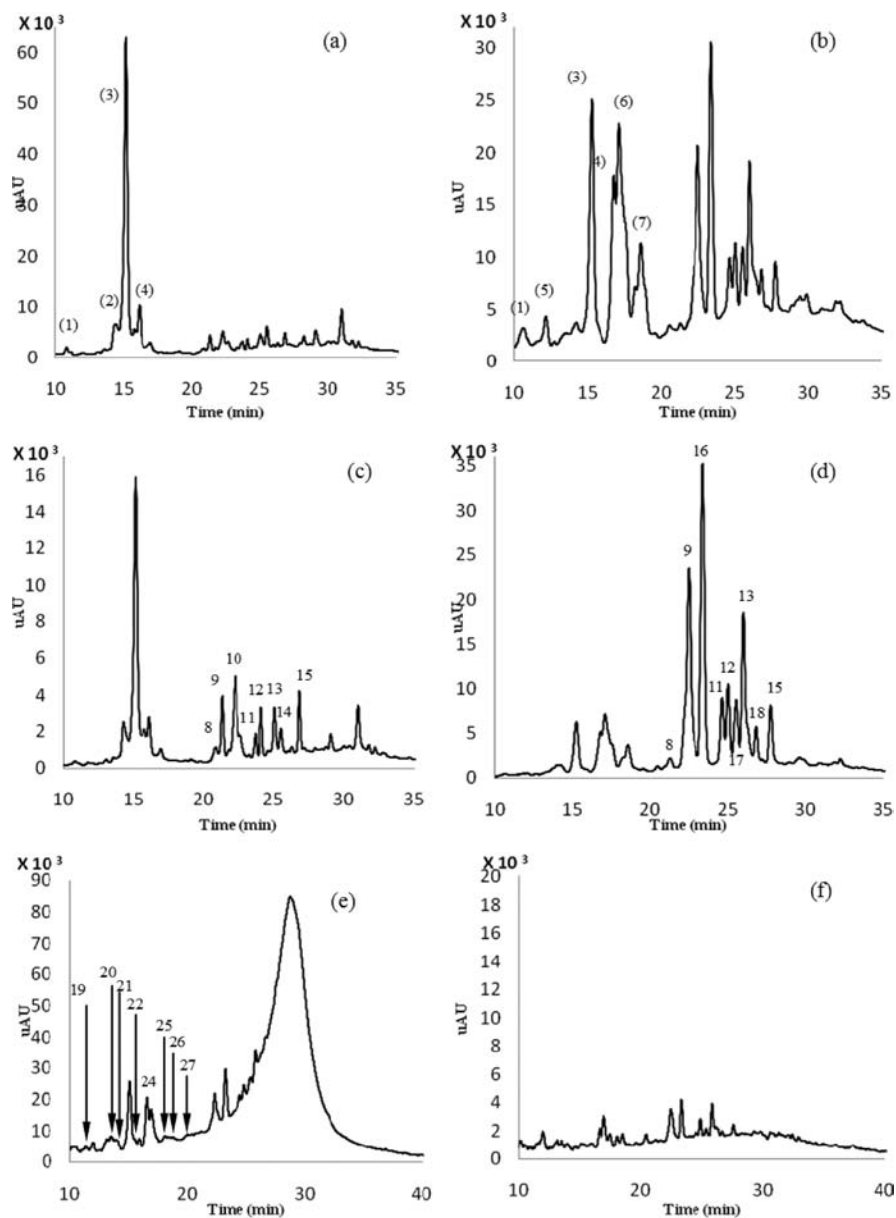

Figure 3. Reversed phase chromatograms of the methanol extracts of the peel of the Deglet Nour variety at different maturity stages: (a) UV chromatogram at 330 nm at the Kimri stage; (b) UV chromatogram at 330 nm at the Khalal stage; (c) UV chromatogram at 360 nm at the Kimri stage; (d) UV chromatogram at 360 nm at the Khalal stage; (e) UV chromatogram at 280 nm at the Khalal stage; (f) UV chromatogram at 280 nm at the Tamar stage.

mentioned in the literature.[10,11] Interestingly, the three regioisomeric shikimic acid esters showed reproducible differences in their MS/MS spectra, allowing their identification as previously described by Jaiswal[21] in maté and by Karakose et al.[22] in stevia leaves. Only the molecular ion of compound 2 showed an intense product ion at m/z 161 [(M − shikimic acid moiety − H_2O) − H]$^-$ and was thus identified as 4-CSA.[21] The MS/MS spectrum of compound **3** clearly revealed a product ion at m/z 317 [(M − H_2O) -H]$^-$, which allowed its identification as 5-CSA.[21] This product ion was not observed in compound **4**, which was therefore identified as 3-CSA.[21]

We noted that the order of elution of caffeoylshikimic acid regioisomers tentatively identified on the C18 column in our HPLC conditions (4-CSA > 5-CSA > 3-CSA) was slightly different from that found in previously published data, where 4-CSA was eluted after 5-CSA.[21] This reversal can be attributed to the nature of the column and the solvents used.

The compounds corresponding to peaks 1 and 5 showed deprotonated molecules [M − H]$^-$ at m/z 497 (Figure 3b), which was consistent with (i) a caffeoylshikimic acid hexoside or (ii) a dicaffeoylshikimic acid structure. Their UV spectra, similar to that of caffeic acid, could not be used to clearly distinguish between these two hypotheses. However, their early retention times (10.3 and 12.0 min for compounds 1 and 5, respectively) indicate that these compounds are highly polar, which is much more consistent with the presence of a sugar moiety in the structure. In addition, an intense product ion was observed on their MS/MS spectra (Table 1) at m/z 341 (loss of 156 amu corresponding to a shikimic acid moiety). This product ion was absent in the MS/MS spectra of a series of dicaffeoylshikimic acid isomers previously published by

Table 2. Total Flavanol Fraction (Including Catechins and Procyanidin Oligomers and Polymers) in the Stone, Peel, and Flesh of Deglet Nour and Ftimi Dates at the Ripe Stage (Tamar) Determined by RP-HPLC Following Phloroglucinolysis Apply on Fruit Powder Samples

						terminal units				extension units	
						CAT[a]		EC[b]		EC[b]	
variety	tissue	total flavan-3-ols (mg/fruit)	SD[c]	DP*n*	SD[c]	%	SD[c]	%	SD[c]	%	SD[c]
				Kébili							
Deglet Nour	stone	21.8	0.5	7.5	0.4	7.09	0.39	6.23	0.25	86.7	0.63
	peel	52.2	0.2	8.9	0.5	0.62	0.07	10.61	0.69	88.8	0.68
	flesh	105.0	4	33.2	1.0	0.63	0.01	2.39	0.08	97.0	0.09
Ftimi	stone	26.0	2.3	11.5	0.3	4.75	0.33	3.98	0.23	91.3	0.2
	peel	67.7	5.3	20.5	0.7	2.51	0.15	2.37	0.15	95.1	0.18
	flesh	65.2	6.3	29.4	0.3	1.53	0.05	1.87	0.06	96.6	0.03
				Tozeur							
Deglet Nour	stone	25.8	0.9	7.2	0.1	8.43	0.11	5.42	0.12	86.1	0.15
	peel	60.0	0.2	15.5	0.3	0.6	0.12	5.85	0.03	93.5	0.14
	flesh	108.8	6.8	30.1	0.2	0.99	0.04	2.33	0.04	96.7	0.02
Ftimi	stone	25.0	1	12.5	0.3	4.51	0.14	3.5	0.09	92.0	0.19
	peel	58.9	5.3	21.2	1.1	2.38	0.1	2.35	0.13	95.3	0.23
	flesh	68.3	2.1	28.9	0.3	1.56	0.04	1.91	0.07	96.5	0.04

[a]CAT, (+)-catechin. [b]EC, (−)-epicatechin. [c]SD=, standard deviation ($n = 3$).

Gouveia et al.,[23] whereas the presence of m/z 341 ions is consistent with a caffeoyl hexoside as previously published by Bastos et al.[24] Finally, another main product ion at m/z 335 (loss of 162 amu) can be attributed to the loss of a hexoside moiety. The compounds corresponding to peaks 1 and 5 were therefore tentatively identified as caffeoylshikimic acid hexosides. Interestingly, greater quantities of these two isomers were observed in the advanced maturity stages. Thus, peak 5 was observed in only the Khalal and Tamar stages. As far as we know, these compounds have never been mentioned before in the polyphenol composition of dates.

Two compounds (peaks 6 and 7, Figure 3b; Table 1) were detected in the Khalal and Tamar maturity stages with retention times of 17.3 and 18.4 min, respectively. According to the mass and UV spectra, these compounds probably correspond to HCA. The deprotonated molecules $[M - H]^-$ at m/z 547 and 709 were consistent with a caffeoylsinapoyl hexose and a dicaffeoylsinapoyl hexose, respectively. Indeed, the MS/MS spectra of the molecular ion m/z 547 (peak 6) showed three main product ions at m/z 385, 223, and 205 corresponding to the loss of a dehydrated caffeoyl moiety (loss of 162 amu), a deprotonated sinapic acid moiety (m/z 223), and a dehydrated sinapic acid (m/z 205), respectively. A similar MS/MS fragmentation pattern was observed for the molecular ion m/z 709 (peak 7) showing major product ions at m/z 547 (loss of 162 amu, corresponding to the loss of the first dehydrated caffeoyl moiety), m/z 385 (loss of the second dehydrated caffeoyl moiety), and m/z 223 and 205 corresponding to deprotonated sinapic acid and its dehydrated form, respectively. As far as we know, this is the first experimental evidence of caffeoyl−sinapoyl conjugates in dates.

Flavan-3-ols. The FA monomers and oligomers were analyzed in the methanol extracts. These compounds were detected in the UV chromatograms at 280 nm (Figure 3e,f). They were also more clearly detected oi the MS extracted ion chromatograms of the corresponding molecular ions in the Khalal and Tamar stages.

The characterization of the total FA class, including monomers and PC oligomers and polymers, was achieved by acidic depolymerization of crude freeze-dried date powders in the presence of an excess of a nucleophile (i.e., phloroglucinol), followed by HPLC-UV analysis of the reaction medium. This method was successfully used in a previous study for the characterization and quantification of condensed tannins in fruits and other plant materials.[16]

(a) Flavanol Monomers and Procyanidin Oligomers. Flavan-3-ol monomers and PC oligomers were clearly detected in the peel and in the stones (Table 1): (+)-catechin (CAT, peak 20), (−)-epicatechin (EC, peak 24), and PCs B1 and B2 (peaks 19 and 21, respectively) were identified by comparison with available standards, whereas other unidentified PC dimer, trimer, tetramer, and pentamer (peaks 22, 25, 26, and 27) were characterized according to their molecular ions $[M - H]^-$ at 577, 865, 1153, and 1441, respectively. In addition, MS/MS spectra of these ions showed the typical fragmentations of PC oligomers, including the retro-Diels−Alder fragmentation of the heterocycles (loss of 152 amu), the loss of the phloroglucinol A-ring (loss of 126 amu), and the rupture of the interflavan linkage (loss of 288 amu) as observed in previously published works.[25] These oligomers were previously detected in dates.[12] In the final maturity stage, PC dimers, trimers, and (+)-catechin were still present in the stones but were no longer detected in the methanol extracts of the flesh and peel.

On the basis of the UV spectrum (a single band at 278 nm), a large peak corresponding to the FA class was clearly observable on the UV chromatogram at 280 nm of the methanol extract of the peel at the Khalal stage (Figure 3e). Interestingly, this peak was no longer present in the Tamar stage chromatogram (Figure 3f). Mass spectrometry analysis of this chromatographic zone (data not shown) revealed a series of ions consistent with polymerized PCs.[26] In particular, ions at m/z 1297, 1585, and 1873 were clearly observable and can be unambiguously attributed to doubly charged molecular ions DP9, DP11, and DP13 as previously described by Guyot et al. in the case of apple PCs.[26] In addition, signals at m/z 1153, 1441, and 1729 are consistent with doubly charged DP8, DP10, and DP12 molecular ions. However, they could also correspond

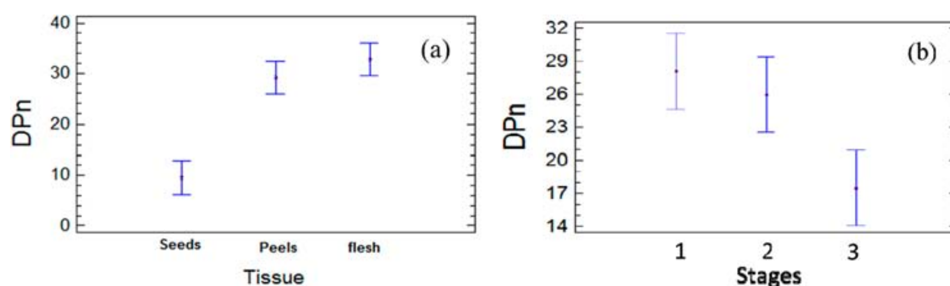

Figure 4. Means and 95% confidence intervals for DP*n* of different tissues at different maturities (a) and at different stages considering all tissues (b). Stages: 1, Kimri; 2, Khalal; 3, Tamar.

to monocharged DP4, DP5, and DP6. As a first interpretation, the absence of this large peak in the Tamar stage could be consistent with a strong decrease in the concentration of PC polymers at the mature stage. However, according to the results of the characterization and quantification of total PCs using phloroglucinolysis (see paragraph below), it could also likely be explained in part by a strong decrease in the extractability of polymerized PCs using methanol for samples at the mature stage. This observation can be related to the loss of astringency of dates in the ripe stage: it is well-known that PCs (i.e., condensed tannins) greatly contribute to astringency (i.e., puckery and dry feeling in the mouth) as a result of their physicochemical aggregation with salivary proteins. If those tannins are strongly linked to the insoluble matrix of the fruit (i.e., cell wall polysaccharides), they are no longer available for association with salivary proteins, which may result in a strong decrease in the astringent taste when fruits are eaten.

(b) Characterization of Total Procyanidins (Condensed Tannins). The acidic phloroglucinol reaction, applied to freeze-dried powders, followed by HPLC analysis of the reaction media, was used to characterize total condensed tannins in date tissues on the basis of their DP*n* and their FA unit distribution. Data concerning the ripe stage (Tamar) of the Deglet Nour and Ftimi varieties are presented in Table 2. PCs were wholly homogeneous, constituted essentially of (−)-epicatechin (EC) representing >97% of total FA units. EC was the only unit found as extension units in all tissues. EC and (+)-catechin (CAT) were found as terminal units in the edible parts (peel and flesh), the former generally being in higher proportions. Interestingly, in the stones, CAT contributed more to terminal units with similar or even slightly higher proportions than EC (Table 2). CAT thus reached 8% of total FA units in the stone of the Deglet Nour variety.

At maturity, DP*n* of date PCs varied from 7 to 33 depending on the variety and the tissue zones (Table 2). On the whole, the order of variation in DP*n* values was as follows at maturity: stones < peel < flesh. However, differences in DP*n* between peel and flesh were no longer statistically significant when the data from several maturity stages were combined (Figure 4a). Stone PCs also revealed a lower DP*n* ranging from 7 to 12 depending on the variety (Figure 4a; Table 2). Therefore, considering their relatively high PC concentration (see section on quantitative analysis), date stones could be an interesting waste material for use in the nutraceutical or cosmetics industries.

We clearly noted a significant decrease in DP*n* in the ripe stage compared to the unripe stages (Figure 4b). Considering previous studies showing the tight link between the degree of polymerization of PCs and their involvement in astringency,[27] this observation also helps explain the decrease in astringency of ripe fruits.

Flavonols and Flavones. FO and flavone conjugates at the different maturity stages were characterized by HPLC coupled with UV−visible and MS/MS in negative mode (Table 1; Figure 3c,d). These classes of compounds have already been investigated by Mansouri et al.[10] and Hong et al.[12] using HPLC-ESI-MS/MS in positive mode. In these papers, the partial identification of these molecules in dates at maturity has been clearly discussed on the basis of the molecular ions and the main product ions observed on the MS/MS spectra. These studies revealed that FOs in dates were mainly quercetin and isorhamnetin (quercetin 3′-methylether) glycosides, and flavones were mainly chrysoeriol (luteolin 3′-methylether) glycosides. However, except for the distinction between pentosyl and hexosyl moieties, complete identification of the sugar moiety remains difficult. In addition, the loss of 80 amu was observed for some compounds on the MS/MS spectra and was interpreted as an indication of the presence of a sulfate group in the molecular structure.[12,28]

In the following section, we will only briefly develop the discussion concerning the identification of the compounds already described by Hong et al.[12] Their chromatographic, UV−visible, and MS characteristics are presented in Table 1. More attention will be paid to the appearance or disappearance of those flavonoids in the peel according to the maturity stage and to molecules that have not been previously mentioned in dates until now.

First, acidic hydrolysis was applied to a selected sample (i.e., peel of the Deglet Nour variety at Tamar maturity stage) to obtain information concerning the nature of the flavone and FO aglycones present as conjugates. Three main aglycones corresponding to quercetin ($[M - H]^-$ = 301, RT = 30.4), isorhamnetin ($[M - H]^-$ = 315, RT =35.5), and chrysoeriol ($[M - H]^-$ = 299, RT = 34.8) were thus unambiguously identified on the basis of their retention time, UV−visible spectrum, and MS data using HPLC−UV−visible and mass spectrometry analysis of the hydrolyzed extract by comparison with authentic commercial standards. Traces of kaempferol and luteolin ($[M - H]^-$ = 285, RT = 34.6 min and RT = 30.1 min, respectively) were also detected.

Peaks 9, 12, and 16 (Figure 3c,d) were unambiguously identified as quercetin 3-*O*-rutinoside ($[M - H]^-$ = 609), isorhamnetin 3-*O*-rutinoside ($[M - H]^-$ = 623), and quercetin 3-*O*-glucoside ($[M - H]^-$ = 463), respectively, according to their chromatographic, UV−visible, and MS characteristics by comparison with authentic standards. Quercetin 3-*O*-glucoside was noticeably detected only at the Khalal and Tamar maturity stages (Table 1). Peak 13 showing a molecular ion at m/z 477 and a main product ion at m/z 315 was partially identified as an

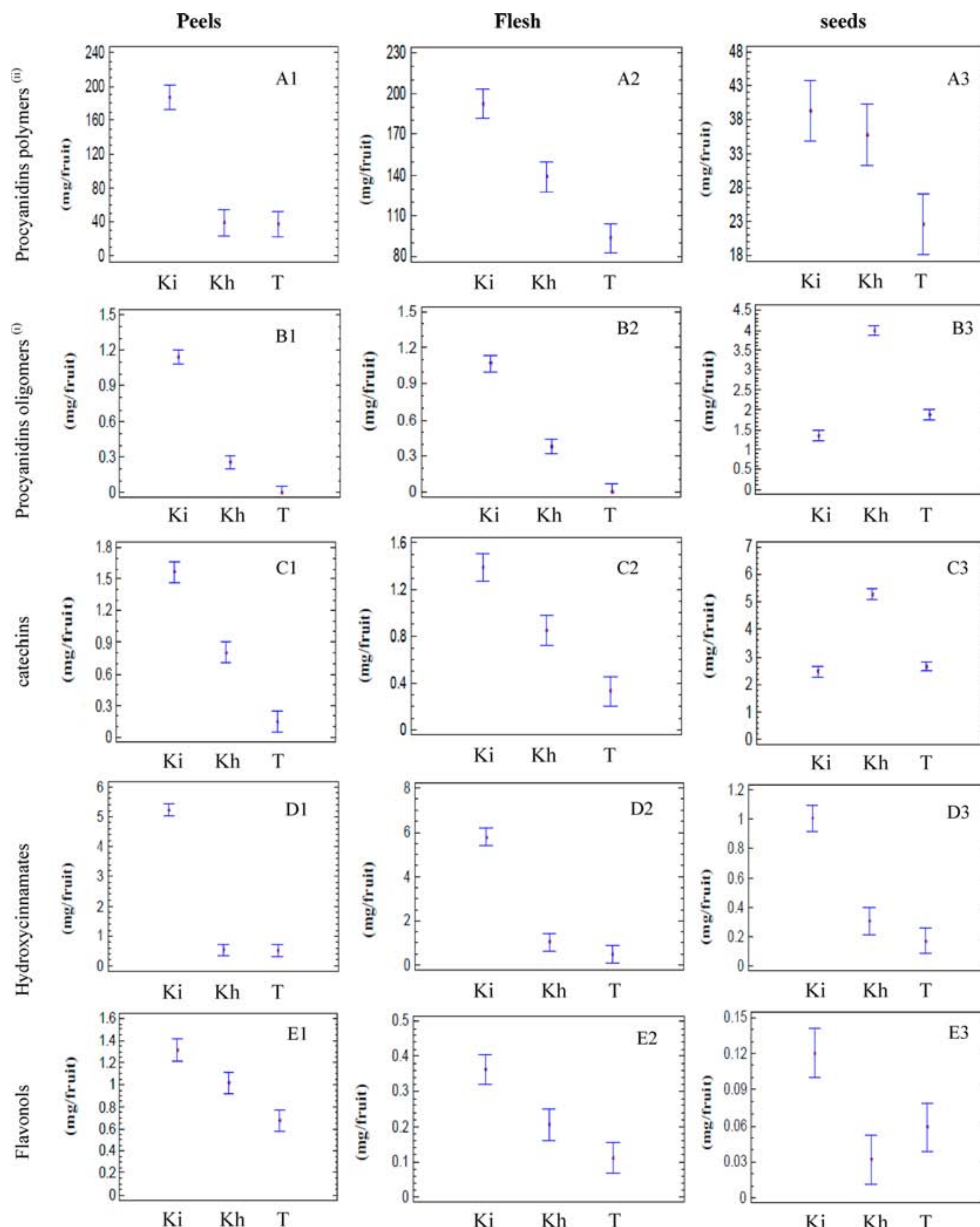

Figure 5. Means and 95% confidence intervals for phenolics class (mg/fruit) in dates at different maturity stages in the peel, flesh, and stone. Ki, Kimri stage; Kh, Khalal stage; T, Tamar stage. (i) PC dimers, trimers, and tetramers; (ii) the rest of the PCs (polymers).

isorhamnetin hexoside. The isorhamnetin moiety was confirmed by observing the close similarity between the 477→315 MS^3 spectrum and the m/z 315 MS^2 spectrum of the isorhamnetin standard (data not shown).

HPLC peaks showing MS characteristics similar to those found for peaks 9 and 12 have already been discussed by Hong et al.,[12] and a similar compound corresponding to peak 16 was described by Mansouri et al.[10] However, as far as we know, this is the first time they have been confirmed in dates by an authentic standard.

Peaks 8 and 10 (RT = 20.7 and 22.1, Figure 3c) revealed molecular ions at m/z 689 and 543, respectively. Their MS/MS spectrum showed a main product ion at m/z 301, which was consistent with quercetin conjugate molecules. Their respective secondary product ions at m/z 609 and 463 (loss of 80 amu) were consistent with the presence of a sulfate group in these molecules. Because no standards were available for comparison, these compounds were partially identified as quercetin rhamnosyl-hexoside sulfate (peak 8) and quercetin hexoside sulfate (peak 10).

Peaks 14, 17, and 18 all revealed an intense product ion at m/z 299. In all cases, the MS^3 spectrum of this product ion was compared with the MS^2 spectrum of the chrysoeriol molecular ion at m/z 299, revealing that all of these spectra were very similar (data not shown). It was therefore unambiguously concluded that these three compounds were chrysoeriol conjugates. According to their molecular ions at m/z 607 and 461, compounds corresponding to peaks 14 and 18 were

Table 3. Average[a] Concentrations (in Milligrams per Fruit) of Polyphenols in Dates at the Commercial Maturity Stage (Tamar)

	peel	flesh	stone	total
av fresh wt of one fruit	1530 (250)	7760 (921)	910 (82)	10200 (400)
catechin monomers[b]	0.15 (0.06)	0.31 (0.15)	2.67 (0.29)	3.13 (0.50)
procyanidin oligomers[b,d]	0.00 (0.00)	0.00 (0.00)	1.90 (0.26)	1.90 (0.26)
procyanidin polymers[c]	33.6 (26.3)	90.6 (20.9)	22.6 (2.25)	146.8 (49.5)
flavonols[b]	0.67 (0.10)	0.12 (0.07)	0.05 (0.01)	0.85 (0.18)
hydroxycinnamates[b]	0.51 (0.13)	0.46 (0.08)	0.14 (0.02)	1.11 (0.23)
total polyphenols[b]	34.9 (26.4)	91.5 (21.1)	27.4 (2.33)	153.8 (49.8)

[a]Mean and standard deviation (n = 18) of two varieties (Deglet Nour and Ftimi), three geographical origins (Kébili, Tozeur, and Douz), and three replicates of plant material (3 batches of 10 fruits). [b]Assayed by HPLC in methanol extracts. [c]Assayed by HPLC after phloroglucinolysis of crude powders and considering subtraction of oligomers assayed in methanol extracts. [d]From dimers to pentamers.

partially identified as chrysoeriol rhamnosyl-hexoside and chrysoeriol hexoside, respectively.

The molecular ion of the compound corresponding to peak 17 (m/z 541) revealed a mass difference of 80 amu compared to the molecular ion observed for peak 18. This difference was consistent with the presence of an additional sulfate group and was confirmed by the observation of MS/MS product ions at m/z 461 and 241 corresponding to the loss of the sulfate group and to a sulfated hexose product ion, respectively. The latter was proof that the sulfate group is linked to the hexose moiety. This compound was thus partially identified as a chrysoeriol hexoside sulfate, probably corresponding to the same compounds previously mentioned by Hong et al.[12] under the denomination hexosyl methyl luteolin sulfate.

Compounds corresponding to peaks 11 and 15 (Figure 3c) were tentatively identified as quercetin acetyl-hexoside and isorhamnetin acetyl-hexoside, respectively, on the basis of their corresponding molecular ions at m/z 505 and 519, which revealed a mass difference of 42 amu in comparison with their nonacetylated analogues (peaks 16 and 13, respectively). This was argued by the observation of m/z 301 and 315 as the major product ions on their MS/MS spectrum, which corresponded to the quercetin and isorhamnetin moieties, respectively. Acetylated FOs have been previously characterized in plants.[29] However, as far as we know, this is the first mention of their probable presence in dates.

On the whole, most of the compounds of these flavone and FO classes were detected in the three maturity stages considered. However, some changes were observed for certain compounds. For instance, quercetin pentosyl-hexoside sulfate (peak 8) was detected in the early Kimri stage but was no longer present in the Khalal and Tamar stages. In contrast, quercetin 3-*O*-glucoside (peak 16) and chrysoeriol hexoside (peak 18) were detected only in the more advanced Khalal and Tamar stages.

Quantitative Variability of Phenolic Compounds. This section is devoted to the quantification of the phenolic compounds in dates. Several variation factors, including tissue zone, maturity stage, variety, and geographic origin, were considered. On the basis of their identification criteria (i.e., retention times, UV−visible, and MS spectra) the phenolic compounds were classified and quantified according to five polyphenol classes: HCA, FOs, flavones, catechin monomers, and PC oligomers (i.e., from dimers to tetramers that were discussed in the previous section) in the methanol extracts. Total FAs (including monomers, oligomers, and polymers) were quantified using acidic depolymerization in the presence of phloroglucinol. By subtracting monomers and oligomers, which were quantified in the methanol extract, from the total FAs quantified by phloroglucinolysis directly applied on the crude powder, it was possible to estimate the concentration of the PC polymer fraction. Analysis of variance (ANOVA, Table 2) was used for the statistical analysis of the quantitative data. Graphical representations of the data are given in Figure 5.

Concentration of Polyphenols at the Tamar Stage (Commercial Maturity Stage). At commercial maturity, and considering the mean of the two varieties and three geographic origins, the average concentration of total polyphenols in a single fruit was estimated at 154 mg according to HPLC quantification (Table 3). About 82% of this quantity is located in the edible parts (peel and flesh). It is noteworthy to mention that this value is much higher than previously published values usually obtained using the colorimetric Folin−Ciocalteu method and reviewed by Al Farsi and Lee.[1] This review mentioned total polyphenols in dates varying from 194 to 240 mg/100 g of fruit, which corresponds to approximately 19−24 mg per fruit when our own data for the average weight of one fruit at the Tamar stage (i.e., 10.2 ± 0.4 g, n = 18) are taken into account. We consider that our estimation better reflects the real concentration of total polyphenols in dates as phloroglucinolysis−HPLC is the only quantification method that takes into account the nonextractable PCs which represent the major part of polyphenols in dates and which are not quantified when a colorimetric assay is performed on a methanol extract.

First of all, results clearly showed that PCs, and particularly polymers (condensed tannins), are by far the most concentrated phenolic compounds in all tissues in dates at commercial maturity. Thus, considering the edible parts of the fruits (flesh + peel), PC polymers accounted for 80% of the total polyphenols in the fruit, with an average value close to 124 mg per fruit (Table 3). As far as we know, these are the first data showing evidence of this preponderant contribution of highly polymerized condensed tannins to the polyphenol composition of dates at maturity. Evidence of a series of PC oligomers up to heptadecamers has been established previously, but quantitative data were not provided.[12] For comparison, this level of total PCs (i.e., 1.24 g/100 g FW) in the edible parts of dates is very high compared to the level quantified in other fruits such as dessert apples,[30,31] red grapes,[32] or plums[33] using comparable acidic depolymerization−HPLC methods that allow the quantification of polymerized PCs. For instance, even in cider apples, which usually contain high amounts of PCs,[34] the levels of PCs reach only 0.6 g/100 g FW and concentrations of PCs in dessert apples are much lower. In the flesh and in the peel, PC oligomers (here considered up to tetramers) were detected as nonquantifiable traces in the ripe stage using mass spectrometry. In the stones, they only

accounted for a small proportion (<8%) of total PCs. For comparison, Guyot et al.[30] showed that, depending on the variety considered and the tissue zone, the PC dimer B2 accounted for 16−18 and 9−12% of total PCs in the flesh and in the skin of apple, respectively. We suppose that tannins at the ripe stage are strongly associated with the cell wall matrix,[35] which may significantly reduce their bioaccessibility and thus impede the precipitation of the salivary proteins responsible for the astringent sensation in the mouth. It is therefore interesting to note that dates are tannin-rich fruits without the frequently observed drawback of bitterness and astringency. Interestingly, FAs are also highly concentrated in the stones with average catechin and total PC values close to 2.7 and 24.5 mg, respectively, per stone. This corresponds to approximately 26 g FAs/kg stones. Moreover, it was shown in the section *Characterization of Total Procyanidins* that PCs in stones were not highly polymerized in comparison with those in the skin and flesh, with an average DP*n* close to 10. Therefore, the stones can be considered as interesting waste that could be used on the basis of the PC content.

Compared to FAs, the other polyphenol classes (i.e., HCA and FOs) are present in very low concentrations (Table 3). HCA accounted for <0.8% of total polyphenols in the fruits. They are equally distributed in the peel and flesh with concentrations close to 0.5 mg per fruit and approximately 0.15 mg per fruit in the stones. FOs only represented 0.6% of total polyphenols in the fruits with an average concentration close to 0.9 mg per fruit. The main part (i.e., 80%) is located in the peel, as generally observed in fleshy fruits. The main compound found in this class was isoquercitrin (quercetin 3-*O*-glucoside) with a proportion of 22% of total FOs in the peel.

Analysis of Variance of Polyphenols in Dates. The table showing the results of the ANOVA is presented in the Supporting Information.

Taking into account the three variation factors that have been considered in this study (i.e., variety, tissue zone, and area of production), it appears that the most influential factor was the maturity stage, as highlighted by a high F value ranging from 17.3 to 799 and P values always <0.0005 for all tissue zones and polyphenol classes considered. This effect is discussed in more detail in the following section.

The second most important factor of variability seemed to be the area of fruit production, which appeared to be significant for the FO class in the peel ($F = 52.7$ and $P < 0.0005$). This effect seemed to contribute more than the variety to the overall variability observed for polyphenols in dates. This might be related to differences in the climatic conditions and particularly sun exposure, which can vary depending on the production zone. However, although they appeared to be significant in our data, this effect has to be considered with caution because only one production year was examined in our study. Nevertheless, within the limits of our study, these observations remain valuable to show, for instance, that environmental factors can counterbalance variability related to variety.

Our study did not reveal a marked effect of variety on the polyphenol composition in dates. A significant effect was observed for PC polymers in the skin, but this was not confirmed in the flesh. Higher variability linked to variety was observed for FOs in the three tissue zones and for HCA in the stones. We are conscious that only two varieties were compared in this study, and therefore our data cannot provide an accurate picture of polyphenol variability in relation to variety. Besides, significant differences between varieties have been shown in previous studies.[9]

Effect of Maturity on the Evolution of Polyphenol Concentrations. Figure 5 shows a detailed picture of the polyphenol concentrations at the different maturity stages for the three tissue zones. Data are expressed in milligrams of the considered phenolic class in one fruit.

As a general trend, the results clearly show a strong decrease in polyphenol concentration as a function of maturity (Figure 5). This decrease was particularly obvious in the edible parts (peel and flesh) but was less marked in the stones. Indeed, a 75% decrease in PC polymers (i.e., condensed tannins) was observed in the peel from the Kimri to the Khalal stage, and the concentration remained stable in the final stage of maturity (Figure 5A1). In the flesh, the amount of these compounds decreased linearly, with a 25% loss from the Kimri to the Khalal stage and a similar loss from the Khalal to the Tamar stage (Figure 5A2). In the stones, the decrease in PC polymers was not significant between the first two stages and did not exceed 20% in the final maturity stage (Figure 5A3). On the whole, the same trends were observed for PC oligomers and catechins in the peel and flesh (Figure 5B1,B2,C1,C2). Obviously, this strong decrease in PC concentrations in the edible parts during maturation is likely one of the main reasons explaining the almost complete disappearance of astringency in the ripe fruits. However, condensed tannin concentrations remain high, even at the commercial maturity stage. Therefore, the loss of astringency is also likely explained by a decrease in tannin bioaccessibility (i.e., strong association with the fruit matrix) in the course of fruit ripening, as discussed in the section *Flavanol Monomers and Procyanidin Oligomers*.

A particular evolution was noticeably observed in the stones (Figure 5B3,C3); the results revealed a significant increase in PC oligomer and catechin levels from the Kimri to the Khalal stage, followed by a 60% decrease at the Tamar stage. In comparison with other tissues, the proportion of PC oligomers in the stones is quite large, with percentages of PC oligomers varying from 2 to 5% of total FAs in the Kimri stage, reaching 15% in the Khalal stage and ranging from 5 to 9% in the Tamar stage (Figure 5B3). On the whole, catechin monomers behave as PC oligomers according to the maturation stage. In the peel and flesh, catechin levels decreased linearly (Figure 5C1,C2), whereas their concentrations in the stones first increased between the Kimri and Khalal stages and then decreased at the ripe (Tamar) stage, returning to the same levels as at the Kimri stage (Figure 5C3). Finally, even for the ripe stage, the stone remained the tissue zone containing the highest catechin levels.

Considering the sum of the quantities in each tissue zone, HCA levels did not exceed 12 mg per fruit, and this level was observed only at the unripe Kimri stage. At this stage, most of these compounds were equally distributed in the peel and in the flesh and were in much lower quantities in the stone. With ripening, their levels decreased sharply, on the order of 80% (Figure 5D1,D2,D3), finally reaching no more than 2 mg per fruit at the ripe stage. This phenolic class therefore remains negligible compared to tannins as it did not exceed 2 or 3% of total polyphenols in the fruit.

FOs were found in low quantities in the fruits and were mainly located in the peel. The highest levels were found at the unripe Kimri stage, reaching 1.4 mg per fruit in the peel (Figure 5E1). Their levels then decreased linearly with maturation in all tissues.

To conclude, as far as we know, this is the first study presenting qualitative and quantitative data revealing the major contribution of PC polymers (i.e., condensed tannins) in the polyphenol composition of edible and nonedible tissues in dates. Other phenolic compounds, including catechins, PC oligomers, HCA, FOs, and flavones, were also clearly represented, although they accounted for only a small part of the total polyphenol content. Some compounds, such as caffeoylshikimic acid hexoside, caffeoyl-sinapoyl monohexoside and dihexoside, and acetylated flavonols, were detected and partially identified for the first time in edible date tissues. Major changes in polyphenol distribution and concentrations were observed according to the maturity stages, mostly revealing a significant decrease in polyphenol levels in fruits during ripening and a decrease in the DP*n* of condensed tannins.

Analyses of the stones revealed that this part of the fruit also contained high concentrations of moderately polymerized condensed tannins and could thus be an interesting waste material for use on the basis of its polyphenol content.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Multiple-range analysis of variance (ANOVA) of polyphenol classes in date tissues. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

PC, procyanidin; FO, flavonol; FA, flavan-3-ol; HCA, hydroxycinnamate; DP*n*, average degree of polymerization

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