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# Characterization of different cultivars of Algerian date palm (*Phoenix dactylifera* L.) leaves and pollen by comprehensive two-dimensional liquid chromatography of phenolic compounds extracted with different solvents

Ourida Benouamane [a,d], María Vergara-Barberán [b,*], Abdelaziz Benaziza [d], María Celia García-Alvarez-Coque [b], Ernesto Simó-Alfonso [b], Bernard China [c], María Jesús Lerma-García [b]

[a] Center of Scientific and Technical Research on Arid Region (CRSTRA), Campus of Mohamed Khider University, Biskra, Algeria
[b] Department of Analytical Chemistry, University of Valencia, C/ Dr. Moliner, 50, 46100 Burjassot, Valencia, Spain
[c] Belgian Health Institute, Quality of the Laboratories, Brussels, Belgium
[d] Department of Agronomic Sciences, University of Biskra, Algeria

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ABSTRACT

The polyphenolic composition of Algerian date palm (*Phoenix dactylifera* L) leaves (in Hamray, Safray, Ghars, Horra, Deglet Nour (DN) cultivars), and pollen (in DN cultivars), were investigated for the first time in these cultivars. The polyphenolic fractions were extracted using three solvents (acetone, methanol and ethanol), and characterized in terms of antioxidant activity and contents of total polyphenols (TPC), flavonoids (TFC), and tannins (TCTC). All the extracts exhibited high antioxidant activity and TPC, TFC and TCTC values, DN being the cultivar that provided the best results without significant differences among the three solvents. When the extracts were analyzed by HPLC-MS, 21 and 23 phenols were identified in leaf and pollen extracts, respectively. Furthermore, polyphenolic fingerprints of leaf extracts were obtained by comprehensive two-dimensional liquid chromatography (LC × LC), which made it possible to discriminate samples according to their cultivar by linear discriminant analysis.

## 1. Introduction

Date palm (*Phoenix dactylifera* L.), a monocotyledonous woody perennial belonging to the *Arecaceae* family, is one of the oldest and most popular fruit trees of arid and semiarid regions of the planet, which is particularly abundant in the Gulf countries of the Middle East [1]. Its cultivation is particularly important for the population living in the Algerian Sahara [2]. In recent years, Algeria has been the fourth world producer of date palm fruits and the ninth in terms of world exporter [3].

Date palm fruits are known for their multiple beneficial effects, due to their high phenolic content and antioxidant capacity [4]. There are several published studies related to the phenolic composition of some date palm seed varieties [5–9], and fruits [2,8,10–12]. However, little information has been published related to the composition of polyphenols in other parts of the trees or their by-products (such as date palm leaves and pollen) [7,10,13–15]. These by-products have also been used in pharmaceutical products and formulations, since the leaf extracts exhibit antifungal, antioxidant, anti-inflammatory and anti-diabetic activities [14]. In addition, more recently, date palm leaf extracts have been used as functional foods, being incorporated to animal diets resulting in numerous benefits [16]. Therefore, it is interesting to study the composition of these by-products, in order to obtain more information regarding the composition of bioactive compounds present in both edible and non-edible parts of date palm trees.

There are several studies published in literature related to the total polyphenol content and antioxidant activity of date palm leaves and pollen [7,8,10,13,17]. Specifically, these studies correspond to varieties grown in countries such as Algeria [7], Saudi Arabia [8], Palestine [10], Tunisia [13], and Egypt [17]. Regarding Algerian varieties, only two leaf varieties (Deglet Nour (DN) and Hamraya (HM)) and one pollen variety (DN) have been previously characterized, which generates a lack of information on other important cultivars located in Algeria.

According to these reports, the most abundant phenols present in date palm leaves are flavonoids (such as catechin and epicatechin) and phenolic acids (protocatechuic and coumaric acids, among others) [8,10,18], while pollen is mainly constituted by a high amount of fatty

* Corresponding author.
*E-mail address:* maria.vergara@uv.es (M. Vergara-Barberán).

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acids (such as palmitic, linoleic and linolenic) [7] and flavonoids [4,10].

To determine polyphenols, the most usual technique is high performance liquid chromatography (HPLC), mainly coupled to mass spectrometry (MS) detection [5,6,9,10], although gas chromatography coupled to MS has been also used to characterize phenolic compounds [19,20]. However, in the last years, two-dimensional liquid chromatography (2D-LC) has appeared as a potential alternative, since it is able to detect more peaks with regard to one-dimensional LC, and does not imply very sophisticated instrumentation (such as mass spectrometers) [21]. In the comprehensive mode, also called LC × LC, discrete portions of the first dimension ($^1$D) effluent are collected over a short period of time, and transferred one at a time to a column in the second dimension ($^2$D), where the system selectivity must be different to obtain good orthogonality [22,23]. The $^2$D profiles can reveal similar peak patterns for specific analyte functional groups. It has been shown that the use of 2D-LC can be a useful tool to establish polyphenol profiles of grapevine canes [24], guabiroba [25], olive leaves and pulps [21], and *Brassica juncea* [26]. Moreover, the $^2$D polyphenolic profiles can be used in combination with statistics to classify samples according to different categories, as demonstrated by Vergara-Barberán et al., [21]. These authors constructed a linear discriminant analysis (LDA) model able to distinguish olive leaves and pulp extracts according to their cultivar, using polyphenolic profiles obtained by LC × LC. The results derived from this work revealed that $^2$D polyphenolic fingerprints are characteristic of each genetic variety, being this methodology easily transferable to other food or agronomic matrices.

The objective of the current study was to explore the phenolic composition of Algerian date palm leaves and pollen coming from five different cultivars (HM, Safray (SF), Ghars (GH), Horra (HR), and DN), most of them for the first time. The possibility of discriminating the different samples according to their cultivar is also evaluated. For this purpose, three different solvents were used to carry out the extraction of phenolic compounds from both date palm leaves and pollen. Then, the contents of total phenols, flavonoids and tannins, and the antioxidant capacity of the samples were determined and compared. Next, the identification and quantification of the main phenols present in the samples was carried out by means of HPLC-MS. Additionally, an LC × LC method was developed to obtain polyphenolic fingerprints of the different extracts of date palm leaves, in order to classify them according to their cultivar using LDA.

## 2. Materials and methods

### 2.1. Plant materials

Fresh leaves of five different cultivars (HM, SF, GH, HR, DN), from six different trees each, were collected and analyzed separately. All these 30 trees were located in the "El Firdous Garden" (Biskra, South-East of Algeria), all samples being collected in the spring of 2019/2020. When received, 25 leaves from each tree were pooled, and treated as follows: washed with distilled water, dried in an oven (UN110 Memmert, Germany) at 50 °C for 24 h and ground until obtaining a fine powder using a mechanical grinder (IKA werke, GMBH and Co., Germany). The resulting powder was stored at –20 °C until the extraction was carried out. Therefore, a total of 30 leaf powder samples (6 trees × 5 cultivars) were obtained for further analysis.

Pollen samples were collected from fresh male spathes of six different trees of DN cultivar, in the same place indicated above. Four spathes were collected for each tree, the four been shaken together inside a plastic bag to recover the pollen powder. The pollen was then dried and stored as specified above for leaf samples. Therefore, a total of six pollen samples were obtained.

### 2.2. Chemicals

Acetonitrile (ACN), methanol (MeOH), ethanol (EtOH), and acetone were obtained from LabKem (Barcelona, Spain). 2,2-Diphenyl-1-picrylhydrazyl (DPPH, 95 % purity) and aluminum chloride ($AlCl_3{\cdot}6H_2O$) were supplied by Alfa Aesar (Massachusetts, USA). Folin Ciocalteu phenol reagent, sodium carbonate ($Na_2CO_3$), formic acid (FA), trifluoroacetic acid (TFA), gallic acid, quercetin, vanillin and (+)-catechin, hydrate hydroxytyrosol, p-hydroxybenzoic acid, cinnamic acid, protocatechuic acid, p-coumaric acid, caffeic acid, chlorogenic acid, apigenin, kaempferol, dihydrokaempferol, rutin, and naringin were supplied by Sigma-Aldrich (St. Luis, MO, USA). 3-Caffeoylshikimic acid was from ChemFaces Biochemical Co. (Hubei, China). Hydrochloric acid (HCl, 37 % purity) was obtained from Panreac (Barcelona). Deionized water was collected from a B30 water purification system (Adrona, Riga, Latvia). All chemicals used were of chromatography quality grade.

### 2.3. Extraction of phenolic compounds

The extraction of phenolic compounds from the 36 samples considered in this study (30 leaves and 6 pollen samples) was carried out according to the maceration method adopted by Dzoyem et al., [27], using three different solvents: acetone, MeOH and EtOH. Briefly, a 10 g portion of each dry powdered plant material was extracted with 100 mL of the corresponding solvent, magnetically stirred for 30 min at room temperature, and then centrifuged at 4000 × g for 10 min (Nahita 2749 centrifuge, AUXILAB S.L, Spain). The residue was re-extracted two additional times under the same conditions, the three obtained supernatants being combined. After filtering with Whatman No.1 filter paper, the filtrate was vacuum dried at 50 °C in a rotary evaporator (Buchi R-100, Flawil, Switzerland). Finally, the dry crude extract was weighed and the yield of soluble phenols, calculated as:

$$Yield\ (\%) = (W_1 \times\ 100)/W_2$$

where $W_1$ was the weight of the extract with the different solvents after evaporation, and $W_2$ was the dry weight of the sample of fresh leaves or pollen. The yields obtained for the samples considered are included in Table 1.

The same protocol was also applied to spiked dry powdered leaf and pollen plant materials, in order to evaluate extraction recovery. For this purpose, a proper amount of each one of the polyphenol standard solutions included in Table S1 were added to 10 g of both materials (leaf and pollen) to obtain a final concentration of ca. 10 mg·L$^{-1}$. Recovery values were calculated considering the polyphenol content naturally present in the extract, the spiked content and the total amount detected. For date palm leaf, the recovery values were comprised between 88.8 and 97.6 %, while for date palm pollen the recovery interval ranged from 85.4 to 96.4 %.

For further analysis, 10 mg of dry crude extract was dissolved in 10 mL of MeOH, except for chromatographic analysis, in which 3 mg of each extract was dissolved in 200 μL of MeOH. Finally, all extracts were filtered through 0.22 μm Nylon syringe filters.

### 2.4. Determination of radical scavenging activity (DPPH)

Radical scavenging activity was evaluated using the DPPH assay according to the method referred by Karra et al., [15]: 500 μL of extract at different concentrations (2–50 and 200–1000 μg mL$^{-1}$ for leaf and pollen samples, respectively) were added to 375 μL of EtOH and 125 μL of DPPH solution (0.02 % in EtOH) as free radical source. After 60 min of incubation, the absorbance of the remaining DPPH radical (inhibition percentage, IP) was measured at 517 nm using a Hach Langue DR6000 UV–vis spectrophotometer (Düsseldorf, Germany), with IP calculated as:

$$\mathrm{IP}\,\% = \frac{(A.blank - (A.sample - A.control))}{A.blank} \times 100$$

where *A.blank* is the absorbance of 500 μL of MeOH, 125 μL of DPPH-EtOH solution and 375 μL of EtOH; *A.sample* is the absorbance of 500

**Table 1**
Yield of soluble phenolics, antioxidant activity ($IC_{50}$), total phenolic content (TPC), total flavonoid content (TFC) and total condensed tannins' content (TCTC) of date palm leaves and pollen extracts from different cultivars extracted using different solvents.

| Type | Variety | Extraction solvent | Yield of soluble phenolics* (%) | $IC_{50}$ (μg $mL^{-1}$) | TPC (mg GAE/g DW) | TFC (mg QE/g DW) | TCTC (mg CE/g DW) |
|---|---|---|---|---|---|---|---|
| Leaves | HM | Acetone | 13.9 ± 0.9 | 17 ± 1.2 aA | 85 ± 5 aA | 90 ± 2 aA | 520 ± 20 aA |
| | SF | | 15.1 ± 1.0 | 23.7 ± 0.9 aA | 94 ± 5 aA | 96 ± 4 aA | 158 ± 4 aB |
| | GH | | 12.7 ± 0.7 | 21.0 ± 0.8 aA | 70 ± 4 aAB | 73 ± 2 aB | 184 ± 6 aB |
| | HR | | 16.8 ± 1.2 | 40.6 ± 0.8 aB | 55 ± 4 aB | 62 ± 2 aB | 238 ± 12 aC |
| | DN | | 13.1 ± 0.6 | 9.5 ± 0.3 aC | 120 ± 6 aC | 93 ± 4 aA | 480 ± 20 aA |
| Pollen | DN | | 21 ± 2 | 500 ± 30 a | 20 ± 4 a | 12.7 ± 1.2 a | ND |
| Leaves | HM | Methanol | 16.2 ± 0.8 | 17.6 ± 1.6 aA | 172 ± 18 bA | 50.1 ± 1.3 bA | 200 ± 11 bA |
| | SF | | 12.5 ± 1.8 | 18.64 ± 0.16 aA | 180 ± 40 bA | 68 ± 2 bA | 114 ± 5 bB |
| | GH | | 14.3 ± 1.6 | 20.92 ± 0.10 aA | 79 ± 12 aB | 64 ± 2 aA | 151 ± 8 bAB |
| | HR | | 15.3 ± 1.2 | 45.6 ± 0.8 aB | 59 ± 9 aB | 24.0 ± 1.3 aB | 44 ± 2 bC |
| | DN | | 11.9 ± 0.9 | 12.2 ± 0.2 aA | 170 ± 20 bA | 61.9 ± 1.7 bA | 244 ± 13 bA |
| Pollen | DN | | 18.2 ± 1.3 | 3600 ± 900 b | 7 ± 0.9 b | 7.2 ± 1.2 b | ND |
| Leaves | HM | Ethanol | 15.1 ± 1.3 | 13.7 ± 1.6 aA | 69 ± 9 aA | 47 ± 3 bA | 211 ± 8 bA |
| | SF | | 15.6 ± 1.4 | 18.3 ± 0.4 aA | 56 ± 5 aA | 75 ± 3 bB | 163 ± 11 bA |
| | GH | | 14.4 ± 0.6 | 16.6 ± 0.8 aA | 71 ± 9 aA | 71 ± 3 aB | 180 ± 7 cA |
| | HR | | 13.8 ± 0.7 | 45 ± 3 aB | 55 ± 4 aA | 45 ± 2 aA | 106 ± 9 cB |
| | DN | | 18.1 ± 1.0 | 13.7 ± 0.5 aA | 89 ± 5 cA | 60 ± 2 bAB | 301 ± 15 aC |
| Pollen | DN | | 20 ± 2 | 2200 ± 300 b | 13 ± 2 c | 5.2 ± 0.9 b | ND |

*Yield calculated as the mean of 6 extracts for each type of sample.
Values (mean ± standard deviation, n = 18) followed by different lowercase letters between extracts obtained with different solvents for the same variety are significantly different ($p < 0.05$).
Values (mean ± standard deviation, n = 18) followed by different capital letters between extracts obtained from different varieties using the same solvent are significantly different ($p < 0.05$).

μL of extract, 125 μL of DPPH-EtOH solution and 375 μL of EtOH, and *A. control* is the absorbance of 500 μL of extract with 500 μL of EtOH. The antioxidant activity was expressed as $IC_{50}$ (sample concentration that caused a 50 % inhibition of the DPPH radical) [28]. This value was calculated from the graph obtained by plotting the IP against the concentration of the extract. For all samples, the assay was performed in triplicate.

### 2.5. Determination of total phenolic content (TPC)

TPC was determined according to the Folin-Ciocalteu method described by Teow et al., [29], with slight modifications. An aliquot (500 μL) of each extract was diluted with 5 mL of distilled water. Next, 0.5 mL of Folin-Ciocalteu reagent was added and the mixture was allowed to react at room temperature for 3 min. Afterwards, 0.5 mL of 7.5 % $Na_2CO_3$ was added, and the mixture was incubated in the dark at room temperature for 60 min. Then, the absorbance was measured at 760 nm. A gallic acid standard calibration curve (linear range 0.05–0.4 mg $mL^{-1}$) was used to obtain the TPC value. The results were expressed as mg of gallic acid equivalents per g of dry weight extract (mg GAE/g DW). All extracts were measured in triplicate.

### 2.6. Determination of total flavonoid content (TFC)

TFC was determined following the colorimetric assay described by Gursoy et al., [30] with few modifications. Briefly, 0.5 mL of each extract was mixed with 0.5 mL of MeOH; then, 1 mL of $AlCl_3{\cdot}6H_2O$ (2 % dissolved in MeOH) was added, and the resulting mixture was left to stand for 10 min in the dark at room temperature. The absorbance was measured at 415 nm against a blank sample prepared with 1 mL of MeOH and 1 mL of $AlCl_3{\cdot}6H_2O$ solution. TFC was determined using a quercetin standard calibration curve (linear range 0.01–0.05 mg $mL^{-1}$). Results are reported as mg quercetin equivalents per g of dry weight extract (mg QE/g DW). All extracts were measured in triplicate.

### 2.7. Determination of total condensed tannin content (TCTC)

TCTC was estimated using the method adopted by Heimler et al., [31] with few modifications. In this method, 400 μL of each extract was mixed with 3 mL of 4 % vanillin solution (prepared in MeOH) and 1.5 mL of 37 % HCl. The resulting mixture was incubated for 15 min in the dark, and the absorbance was measured at 500 nm. A (+)-catechin calibration curve (linear range 0.1–0.4 mg $mL^{-1}$) was used to estimate the amount of total condensed tannins, which was expressed as mg of (+)-catechin equivalents per g of dry weight extract (mg CE/g DW). All extracts were measured in triplicate.

### 2.8. Statistical analysis

Different statistical techniques, such as one-way ANOVA and

Pearson's correlations, were carried out to evaluate the data obtained from the different cultivars and extraction solvents. Both studies were performed using the Statistical Package for the Social Sciences (SPSS, version 19.0, Chicago, IL, USA). Differences were considered statistically significant for a level of significance $p \leq 0.05$.

### 2.9. HPLC-MS analysis

Phenolic compounds in the studied samples were tentatively identified by HPLC-MS analysis, performed on an Agilent 1260 LC Infinity chromatograph (Agilent Technologies, Waldbronn, Germany) and an AB SCIEX TripleTOF$^{TM}$ 5600 system (AB SCIEX, Foster City CA, USA). The chromatographic conditions used for the polyphenolic separation were adapted from the literature [32]. Specifically, an Acquity UPLC BEH C18 column (2.1 mm × 50 mm i.d., 1.7 μm) (Waters, Barcelona) and a gradient of water (A) and MeOH (B), with 0.1 % (*v/v*) FA, were used. A gradient program was applied by varying the proportions of A and B as follows: 0–2 min, 10 % B; 2–13 min, an increase of B to 100 %; 13–15 min, 100 % B; finally, the column was equilibrated with the initial composition for 15 min. Column temperature was maintained at 25 °C, with a flow rate of 0.4 mL min$^{-1}$, and an injection volume of 5 μL.

The MS system operated in negative ion mode within the range of *m/z* 80–1200 using the acquisition method with survey scan type (TOF-MS) and dependent scan type (product ion) with information dependent acquisition (IDA). MS conditions were as follows: collision energy, −50 V; declustering potential, 90 V; ion spray voltage, –4200 V; ion source gas 1 and 2, 50 psi; curtain gas, 25 psi; drying temperature, 400 °C; time of accumulation, 100 ms. Automated calibration was performed using an external calibrant delivery system (CDS). HPLC-MS data were processed using the PeakViewTM software (AB SCIEX). The polyphenol standard solutions included in Table S1 were prepared in methanol and analyzed by HPLC-MS to obtain calibration curves in the range of 0.005–40 mg L$^{-1}$. The limit of detection (LOD) and limit of quantification (LOQ) of the different analytes determined by HPLC-MS method were comprised between 0.9-1.6, and 3–5 μg L$^{-1}$, respectively.

### 2.10. LC × LC analysis

For the first dimension ($^{1}$D), a 1260 series HPLC system (Agilent Technologies) was used, equipped with an autosampler, binary pump, online vacuum degasser, thermostatic column compartment and DAD. The $^{1}$D column was connected to the inlet of an 8-port/2-position switching valve (1290 series, Agilent Technologies), which allowed the injection of sample fractions (two identical 40 μL sampling loops) into the second dimension ($^{2}$D), consisting of an Agilent 1290 series HPLC system, equipped with a binary pump, degasser, thermostatic column compartment, and DAD.

In the optimized LC × LC method, polyphenolic compounds were separated in $^{1}$D using an Ascentis$^{TM}$ RP-amide column (100 mm × 2.1 mm i.d., 3 μm, Análisis Vínicos, Tomelloso, Spain). The gradients were obtained by mixing water (A) and ACN (B), each containing 0.14 % (*v/v*) FA. The optimized $^{1}$D gradient was as follows: 0–50 min, 5–40 % B; 50–65 min, 40–70 % B, 65–66 min, 70–100 % B; 66–80 min, 100 % B. Finally, a re-equilibration step was applied during 10 min to reach the initial conditions. The injection volume was 10 μL, column temperature was fixed at 40 °C, and the flow rate was set at 0.2 mL min$^{-1}$. For $^{2}$D, a Zorbax Eclipse Plus C18 column (50 mm × 3 mm i.d., 1.8 μm, Agilent, USA), and gradients of water (A) and ACN (B), containing both 0.05 % TFA (*v/v*) were used. The elution was performed with a shifted gradient. Column temperature was 50 °C, and the flow rate was set at 2.5 mL min$^{-1}$. The valve was switched automatically after each 12-second modulation cycle. UV detection was performed at 280 ± 4 nm and 80 Hz, for both dimensions.

The optimal conditions were obtained after testing several columns in both dimensions. In $^{1}$D: Ascentis$^{TM}$ RP-amide column (100 mm × 2.1 mm i.d., 3 μm, Análisis Vínicos), Kinetex PS C18 (50 mm × 2.1 mm i.d., 2.6 μm, Phenomenex, Torrance, CA, USA), and Kinetex F5 (PFP, 50 mm × 2.1 mm i.d., 2.6 μm, Phenomenex). In $^{2}$D: Kinetex F5 (PFP, 50 mm × 2.1 mm i.d., 2.6 μm, Phenomenex) and Zorbax Eclipse Plus C18 column (50 mm × 3 mm i.d., 1.8 μm).

### 2.11. Acquisition, treatment of raw LC × LC data and construction of LDA models

Data were acquired using an Agilent MSD ChemStation (C.01.07 SR2) and processed with the GC Image LC × LC software (version 2.4, GC Image, LLC, Lincoln, NE, USA). To select the common peaks in different chromatograms, the LC × LC data file (chromatogram) was baseline corrected using the GC Image software. Peak volume ratios were used to build LDA models (using SPSS) able to classify date palm leaves according to their cultivar. LDA is a supervised classification technique, which is considered an excellent tool to obtain vectors at the maximal distance between a set of previously defined categories. Up to $N - 1$ discriminant vectors are created, with $N$ being the lowest value among the number of predictors and date palm categories according to their cultivars.

The selection of predictors for the construction of the LDA models was performed using the SPSS stepwise algorithm, with Wilks' lambda ($\lambda_w$) used as selection criterion. Values of $\lambda_w$ close to zero correspond to well-resolved categories, whereas values near 1 correspond to overlapped categories. According to the stepwise algorithm, a predictor is included in the model if the value of $\lambda_w$ after its inclusion does not exceed a pre-selected value, $F_{in}$ (the input threshold of a comparison of variances test, $F_{test}$). The inclusion of a new predictor modifies the significance of those predictors already present in the model. After the inclusion of a new predictor, a rejection threshold, $F_{out}$, is used to decide whether to remove other predictors from the model, leaving the variance unchanged using the pre-selected $F_{in}$ and $F_{out}$ values. The process concludes when no more predictors are entered or removed from the model. The probability values $F_{in} = 0.001$, and $F_{out} = 0.10$ were adopted.

## 3. Results and discussion

### 3.1. Characterization of date palm leaves and pollen in terms of polyphenolic compounds

Algerian date palm leaves and pollen samples considered in this study were characterized in terms of antioxidant activity, TPC, TFC, and TCTC. The results obtained are summarized in Table 1. All leave extracts exhibited moderate to high antioxidant activity [27], with $IC_{50}$ values between 9.5 ± 0.3 and 45.6 ± 0.8 μg mL$^{-1}$ in the DPPH assay, similar to those previously reported in literature [33]. According to these results, the DN variety is the one that presents the highest antioxidant activity with the three solvents considered (values between 9.5 ± 0.3 and 13.7 ± 0.5 μg mL$^{-1}$), with HR being the variety that provided the lowest antioxidant activity (40.6 ± 0.8 to 45.6 ± 0.8 μg mL$^{-1}$). For the same variety, there are no significant differences ($p < 0.05$) when using the three different extraction solvents. The pollen samples exhibited low antioxidant capacity (500 ± 30 to 3600 ± 900 μg mL$^{-1}$).

Regarding TPC, the leaf extract values were between 55 ± 4 and 180 ± 40 mg GAE/g DW. Among the varieties, DN, SF and HM are the ones that provided the highest TPC values, the HR being the one with the lowest value for all the extraction solvents considered. On the other hand, MeOH is the extraction solvent that gave the best performance for all cultivars. These results are similar to those previously reported for date palm leaves [8,13,14,33,34]. In the case of pollen extracts, lower TPC were observed (7 ± 0.9 to 20 ± 4 mg GAE/g DW) compared to those obtained for the leaves. These results are consistent with those previously published [4].

TFC values of the leaf extracts ranged between 24.0 ± 1.3 and 96 ± 4 mg QE/g DW (see Table 1). Among the different varieties, SF is the one that provided the highest TFC values for the three solvents, being the

amount of flavonoids extracted from this variety using acetone (96 ± 4 mg QE/g DW) significantly higher ($p < 0.05$) than those extracted with MeOH and EtOH (68 ± 2 and 75 ± 3 mg QE/g DW, respectively). For all varieties, acetone was the solvent that provided the highest TFC compared to MeOH and EtOH. The values obtained are higher than those reported by Abuelgassim [14] (1.3 to 2.2 mg QE/g DW), although lower than those published by Kriaa et al., [13] (150 to 320 mg QE/g of plant extract for methanolic extracts). As expected, for pollen extracts, the values were quite low (5.2 ± 0.9 to 12.7 ± 1.2 mg QE/g DW) compared to those obtained for leaf extracts, being similar to those previously reported [4,7].

The TCTC values were between 44 ± 2 and 520 ± 20 mg CE/g DW, which are generally higher than those published by Kriaa et al., [13] for methanolic extracts (59 to 119 mg CE/g plant extract). Among the different cultivars, HM and DN showed the highest values for all solvents (200 ± 11 to 520 ± 20 and 244 ± 13 to 480 ± 20 mg CE/g DW, respectively). Among the different solvents, in general, the best performance was obtained using acetone, followed by EtOH and MeOH. The presence of condensed tannins in the pollen extracts was not detected.

A correlation analysis was carried out between the results obtained for the different tests. When the correlation of the antioxidant capacity ($IC_{50}$ values) with the scores obtained with the other tests (TPC, TFC and TCTC) was evaluated, a good negative correlation was observed, providing Pearson's correlation coefficients ($r$) of –0.497, −0.648 and −0.47, respectively. These values confirm the contribution of phenolics, flavonoids and tannins to the antioxidant activity of date palm leaves and pollen. Finally, the correlation between the content of the different types of compounds studied in this work was also evaluated. In this case, $r$ values of 0.517 (TPC-TFC), 0.433 (TPC-TCTC) and 0.753 (TFC-TCTC) were obtained, showing a fairly strong correlation between the content of these compounds. The results obtained agree with those previously published [13].

Finally, all leaves and pollen extracts were characterized by HPLC-MS. Phenolic compounds identified in both types of samples (leaves and pollen) are listed in Table S2. As seen in this table, a total of 21 compounds were identified in date palm leaves, while the number of compounds in date palm pollen was 23. The compounds identified were mainly phenolic acids (such as p-coumaric and caffeic acids), flavonols and flavones (such as luteolin and rutin), and flavan-3-ols (such as catechin and epicatechin). These results are consistent with those previously published [8,10]. Furthermore, various polyphenolic compounds were quantified in the extracts of date palm leaves and pollen obtained with the three solvents (see Table S1). As observed for all cultivars, in general the best solvent for polyphenol extraction was MeOH followed by EtOH and acetone, but with no significant differences. Also, the polyphenol content in leaf extracts was higher than that found for pollen extracts, as expected considering data previously reported in literature [35].

As far as we are concerned, no work has been published reporting individual polyphenolic contents of date palm leaves. However, the contents found in this work were higher than those reported in date palm fruit and seeds, and in the same order of magnitude as those reported for other types of leaf extracts [36,37].

### 3.2. Selection of LC × LC separation conditions

A methanolic extract of date palm leaves of the GH cultivar was used to optimize the $^1$D and $^2$D separation conditions of the polyphenolic compounds. For this purpose, three stationary phases were tested for the $^1$D dimension: RP-amide, C18 and PFP (see LC × LC Analysis Section). These phases were selected considering that RP-amide [38] and PFP [39] have been previously and successfully used in the analysis of polyphenolic compounds from different food matrices, providing high chromatographic performance due to the effective retention of these compounds via π-π interactions between delocalized electrons in the analytes and the phenyl group in the stationary phase [40]. C18 stationary phases have been also used for the determination of a high number of polyphenols present in different parts of date palms (fruits, leaves, clusters, and pollen) [10,11]. The assayed columns (RP-amide, C18 and PFP) had the same diameter (2.1 mm), but different lengths (100, 50 and 50 mm) and particle sizes (3.0, 2.6 and 2.6 μm), respectively.

For the $^1$D dimension, the mobile phase gradient was optimized for all three tested columns using ACN and water, both containing 0.14 % FA. The optimal gradient was selected considering the largest number of peaks in the chromatograms. The best results found for each of the three tested columns are shown in Fig. S1. As can be seen, the highest performance was achieved using the RP-amide column (Fig. S1A), since a large number of polyphenolic compounds could be detected, followed by the PFP and C18 columns. Then, RP-amide and PFP columns were selected as candidates for $^1$D, while the C18 column was not considered suitable for further LC × LC analysis, as no clear profile was obtained. It is worth mentioning that the slow flow rate used (0.2 mL min$^{-1}$) led to long analysis times (80 min) for all columns.

For the optimization of the $^2$D conditions, the RP-amide and PFP columns (used in $^1$D) were combined with two different columns in the $^2$D dimension (see LC × LC Analysis): Kinetex F5 PFP and Zorbax Eclipse Plus C18, both with sub-micro dimensions, highly recommended to provide fast gradients with high efficiencies [21,41]. The first experiment was performed with the PFP column (2.6 μm) in $^1$D and the C18 (1.8 μm) column in $^2$D (PFP × C18 combination; there were no experiments performed with PFP columns in both dimensions). Flow rates were 0.2 and 2.5 mL min$^{-1}$ for the PFP and C18 columns, respectively. The initial $^2$D shifted gradient is depicted in Fig. 1A together with the resulting $^2$D chromatogram (Fig. 1B). As can be seen in this figure, in the 20–40 min $^1$D region, the peaks were not sufficiently eluted in the 12-seconds $^2$D cycle, and appeared in the middle and upper part of the cycle. However, readjusting the ACN percentage along the $^2$D separation (Fig. 1C), the peaks were properly eluted, appearing in the central part of the $^2$D region. This also allowed the resolution of peaks between 50 and 60 min. Under these optimized conditions, a total of 56 peaks were detected (Fig. 1D). These peaks were selected after subtracting the baseline from the signal, using a threshold of detected peak volume of 25 absorbance units per min$^2$.

Using RP-amide in the $^1$D dimension, the two columns (C18 and PFP) were tested in the $^2$D dimension. The results obtained for the first combination (RP–amide × C18) are shown in Fig. 2. With the initial gradient tested (Fig. 2A), the peaks of polyphenolic compounds between 10 and 30 min ($^1$D) were not well resolved, since they appeared at the top of the LC × LC space, eluting at the beginning of a new $^2$D cycle (see Fig. 2B). Consequently, the $^2$D shifted gradient was adjusted as indicated in Fig. 2C. With these conditions, the analytes were well resolved and distributed throughout the $^2$D region (see Fig. 2D). In this case, a total of 145 peaks were observed.

Finally, the results obtained with the RP-amide × PFP combination are shown in Fig. 3. The initial gradient (Fig. 3A) provided poor separation, since most polyphenolic compounds in the sample coeluted (Fig. 3B). After adjusting the ACN content in the range of 15 to 80 min (Fig. 3C), no improvement was achieved. This combination resulted in the detection of only 90 polyphenols (Fig. 3D).

In view of the above results, the RP-amide × C18 combination (Fig. 2C and D), which provided the best LC × LC separation and the highest number of peaks detected, was selected for a more detailed analysis of the date palm leaf and pollen extracts from the different cultivars.

### 3.3. Polyphenolic fraction profiles of date palm leaves from different cultivars by LC × LC

Different studies have shown that LC × LC chromatographic profiles can be used to discriminate among samples of different cultivars, since they provide a large amount of information on complex matrices

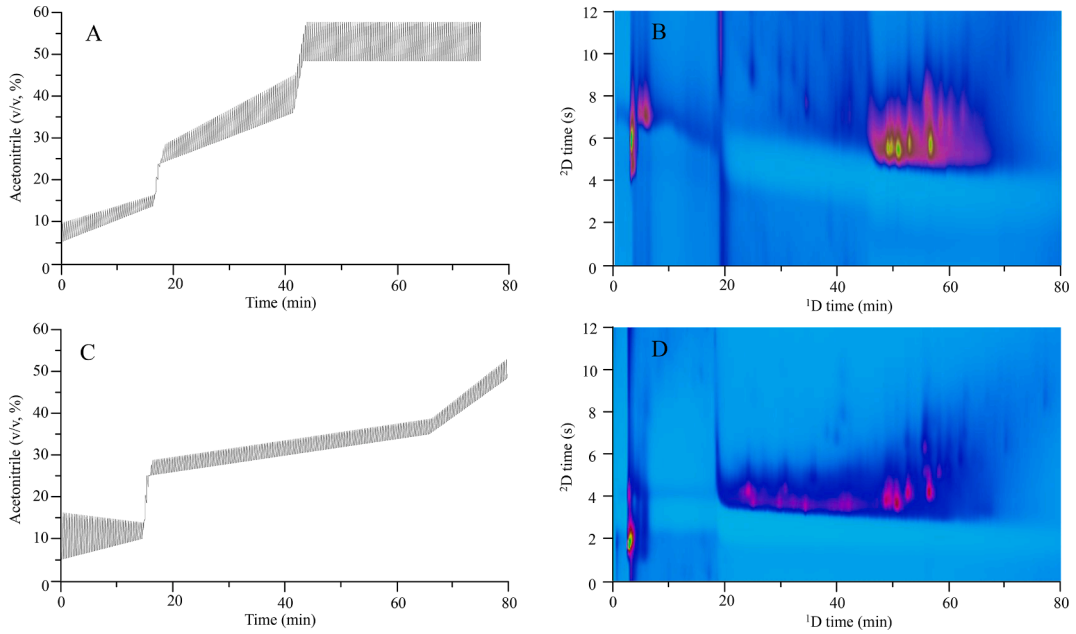


**Fig. 1.** Initial (A) and optimized (C) ACN-water shifted gradients used in the $^2$D separation of polyphenolic compounds in the methanolic extract of GH date palm leaves, using the PFP × C18 column combination, before (B) and after full optimization (D). $^1$D gradient included in Supplementary Material (Fig. S1).

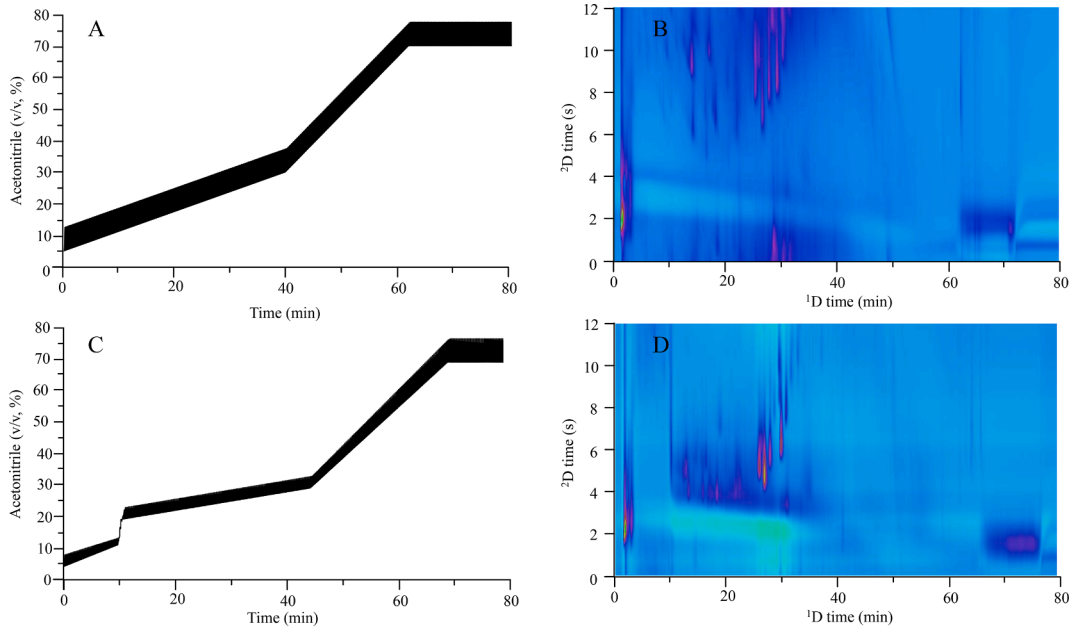


**Fig. 2.** Optimization of the LC × LC separation of polyphenolic compounds in GH date palm leaves, using the RP-amide × C18 column combination. See Fig. 1 for other details.

[21,24,42]. Therefore, the potential of this technique to classify date palm leaves according to their cultivar will be explored. For this purpose, using the optimal combination of LC × LC columns (RP-amide × C18), the polyphenolic extracts of all date palm leaves were analyzed. Representative LC × LC chromatograms, one for each date palm leaf cultivars included in this study, are shown in Fig. 4. The number of peaks detected for each cultivar, using the three different extraction solvents, is provided in Table S3.

### *3.4. Classification of date palm leaves according to their cultivar using LDA*

The peaks detected for all analyzed samples were compared, and those that eluted at the same retention time in both dimensions were selected for the construction of the LDA model. A total of 33 common peaks were found (see those peaks marked with a number in the chromatograms in Fig. 4). For the construction of the LDA model, peak volume ratios were used as predictors instead of the peak volumes, since they have the advantage of highlighting small discrepancies between the components, while accounting to some extent for the variations associated to the extraction process. These volume ratios were calculated by

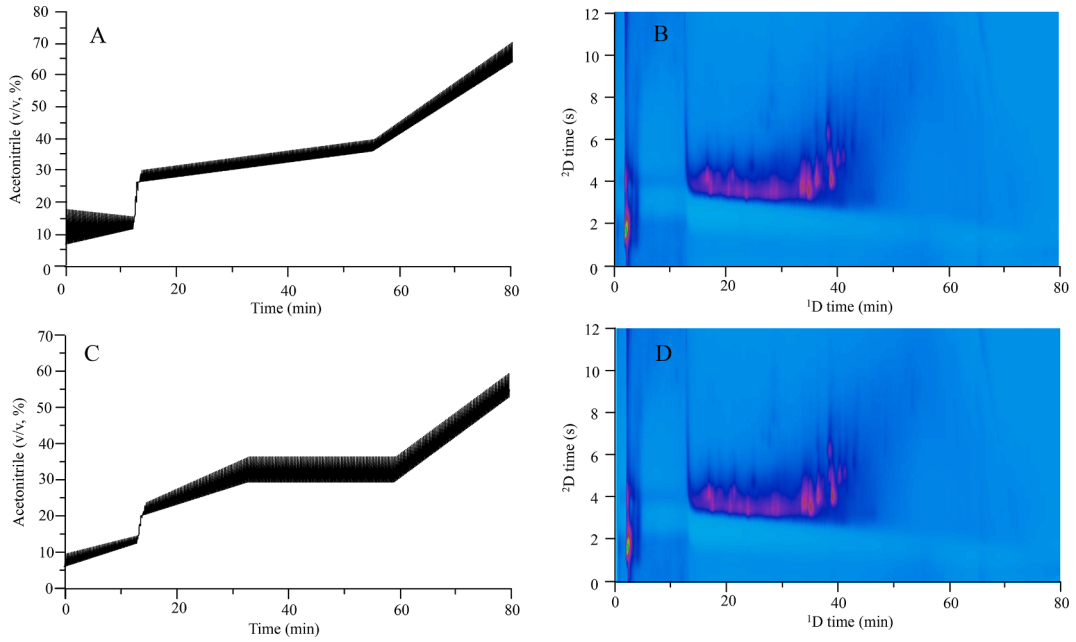


**Fig. 3.** Optimization of the LC × LC separation of polyphenolic compounds in GH date palm leaves, using the RP-amide × PFP column combination. See Fig. 1 for other details.

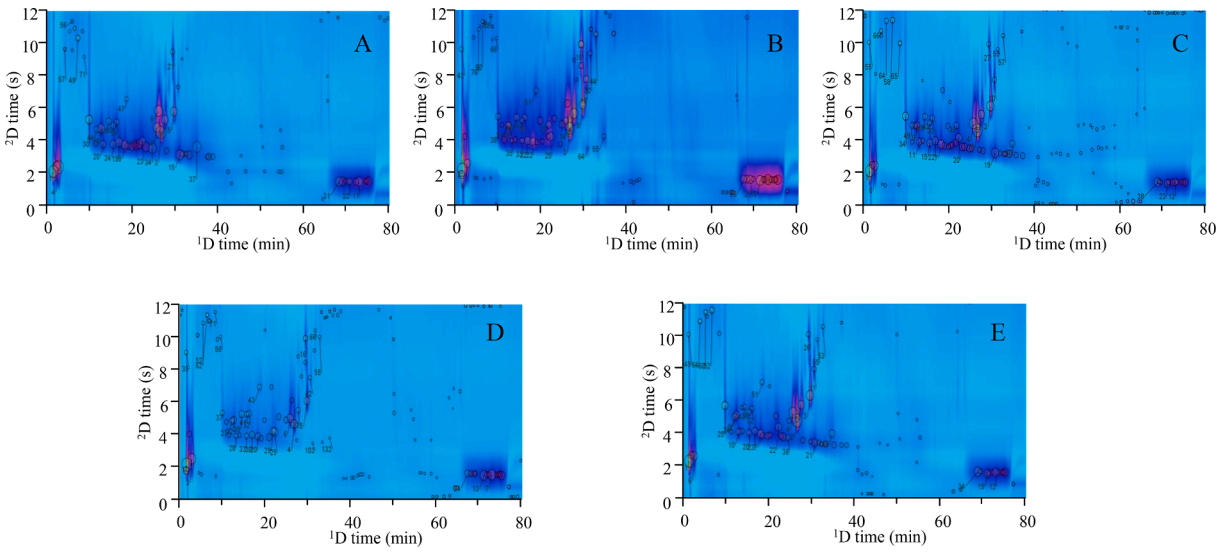


**Fig. 4.** Representative LC × LC chromatograms showing the polyphenolic compound profiles of HM (A), SF (B), GH (C), HR (D) and DN (E) methanolic extracts for date palm leaves. Experimental conditions as indicated in LC × LC Analysis Section.

dividing the volume of each peak obtained from the LC × LC chromatogram by each of the volumes of the other peaks. In this way, and considering that each pair of peaks must be considered only once, the number of non–redundant volume ratios available to be used as predictors was (33 × 32)/2 = 528.

Using the 528 volume ratios as predictors, an LDA model capable of classifying date palm leaves according to their cultivar was built. A data matrix containing 90 objects (5 cultivars × 6 trees × 3 extraction solvents) and the 528 predictors was constructed. A categorical response column was added to this matrix, containing the five categories corresponding to the five cultivars. The matrix was divided into two groups, corresponding to the training and evaluation sets. The training set was made up using 75 objects, which corresponded to 15 randomly selected objects for each cultivar (15 × 5), while the validation set was made up of the remaining 15 objects (3 for each category). Good resolution among the five categories was achieved, when the LDA model was built (Fig. 5, $\lambda_w = 0.116$). The SPSS stepwise algorithm selected a total of 15 predictors (out of 528) as optimal set with the highest discriminant capability. The standardized coefficients of the discriminant functions for these 15 selected predictors are given in Table S4. Finally, regarding the prediction capability of the model, all the objects included in the evaluation set (represented with crosses in Fig. 5) were correctly classified with an assignment probability greater than 95 %.

## 4. Conclusions

The present work has clearly confirmed that all the evaluated leaf and pollen extracts possessed potent antioxidant activity and high TPC, TFC and TCTC, these values being higher for leaves compared to pollen samples. In addition, the results derived from the correlation studies

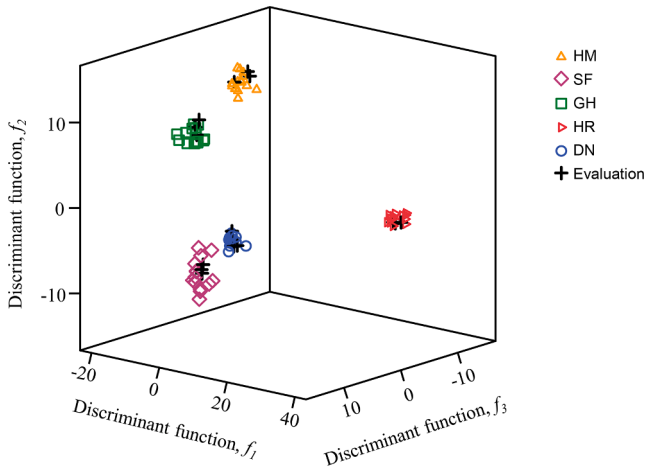


**Fig. 5.** Score plot on an oblique plane of the three-dimensional space defined by the three first discriminant functions of the LDA model, built to classify date palm leaves according to their cultivars. Evaluation set samples are labelled with a cross symbol.

revealed good relationships between the $IC_{50}$, TPC, TFC and TCTC values for all cultivars studied. The highest values of these parameters were obtained in general for the DN cultivar, with no significant difference among the three solvents tested in the extraction of polyphenols.

Using HPLC-MS, a total of 21 and 23 polyphenolic compounds were identified for palm date leaf and pollen extracts, respectively, mainly attributed to phenolic acids, flavonols, flavones and their derivatives. An LC × LC method was optimized in terms of column type and length, particle size and gradient profile, in order to obtain the maximal number of detected polyphenols. Under the optimal conditions, the analysis provided characteristic polyphenolic fingerprints for each cultivar included in this study, being these data useful for the classification of the samples. The comparison of LC × LC polyphenolic profiles showed 33 common peaks for all samples. The peak volume ratios of these polyphenolic compounds were selected and used as predictors for statistical analysis. As a result, date palm leaves from five different cultivars were correctly classified (95 % probability of assignment) using LDA models.

## CRediT authorship contribution statement

**Ourida Benouamane:** Investigation, Formal analysis, Writing – original draft. **María Vergara-Barberán:** Investigation, Formal analysis, Writing – original draft. **Abdelaziz Benaziza:** Conceptualization, Writing – review & editing. **María Celia García-Alvarez-Coque:** Conceptualization, Funding acquisition, Writing – review & editing. **Ernesto Simó-Alfonso:** Conceptualization, Methodology, Validation, Funding acquisition, Supervision, Writing – review & editing. **Bernard China:** Conceptualization, Writing – review & editing. **María Jesús Lerma-García:** Investigation, Conceptualization, Methodology, Validation, Writing – review & editing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

## Acknowledgments

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2022.107874.

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