
Seasonal and Interannual Variability in the Phenolic Content of the Seagrass *Nanozostera noltei*: Characterization of Suitable Candidates for the Monitoring of Seagrass Health

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Supplementary data

S1. Zosteraceae family

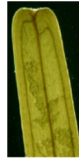
The subdivision of the Zosteraceae family has long been the subject of debate (Jacobs and Les, 2009). Contradictory reports for two, three and four genera have been supported at various times to assemble related species. A discussion still continues about whether to divide the genus *Zostera* into two genera, *Zostera* and *Nanozostera*. *Z. noltei* has been reclassified as *Nanozostera* on the basis of morphological characteristics (Tomlinson and Posluszny, 2001), while Les et al. (2002) proposed maintaining this species in the genus *Zostera*. Based on a combination of morphology and genetic data, the Zosteraceae are now considered to be composed of four described genera: *Phyllospadix*, *Zostera*, *Heterozostera* and *Nanozostera* (Sullivan and Short, 2023). The species is classified as *Nanozostera noltei* (Hornem.) Toml. and Posl., Taxon 50(2): 433 (2001): (2001) in the International Plant Names Index (IPNI; <https://www.ipni.org>). However, the World Register of Marine Species (WoRMS; (<https://www.marinespecies.org>)) still uses the name *Zostera noltei*, Hornem. and considers *Zostera* subg. *Zosterella noltei* Hornemann as an alternate representation, but it considers *Nanozostera noltei* (Hornemann) Tomlinson and Posluszny, 2001 as unaccepted. In addition, caution is advised when reviewing the literature on this species, as the former name, *Zostera nana* Roth., was used up to 1995, and the name *Zostera noltii* is often still used today.



Figure S1. Distribution map of *Nanozostera noltei* Hornem. from IUCN (<https://www.iucn.org>).

S2. *Nanozostera noltei* identification keys (Kuo and den Hartog, 2001)

Leaves, grass: green in color. Blade apex emarginated, often asymmetric, becoming indented in older leaves. Leaves: 6–30 cm long, 0.5–1.85 mm wide with three irregularly spaced veins. Leaf sheath short, 0.54 cm, and open with two membranous flaps. Reproductive shoots lateral. Seeds: 1.5–2 mm long (excluding style), white, and smooth. Rhizome: 0.5–2 mm thick with 1–4 roots per node. Rhizome with fiber bundles in the innermost layers of the outer cortex. Roots are generally in groups of two or three.



blade apex

For this species, chemical variations between populations have made it possible to separate them into three distinct groups and biogeographic regions (Fig. 1 and S2; Grignon-Dubois and Rezzonico, 2018). One is characterized by apigenin 7-sulfate (the eastern part of the Gulf of Cadiz) and one by diosmetin 7-sulfate (the French Atlantic coast and Mediterranean Sea), and the third contained similar quantities of the above two compounds (Mauritania and South Portugal) (MGD chemotypes).

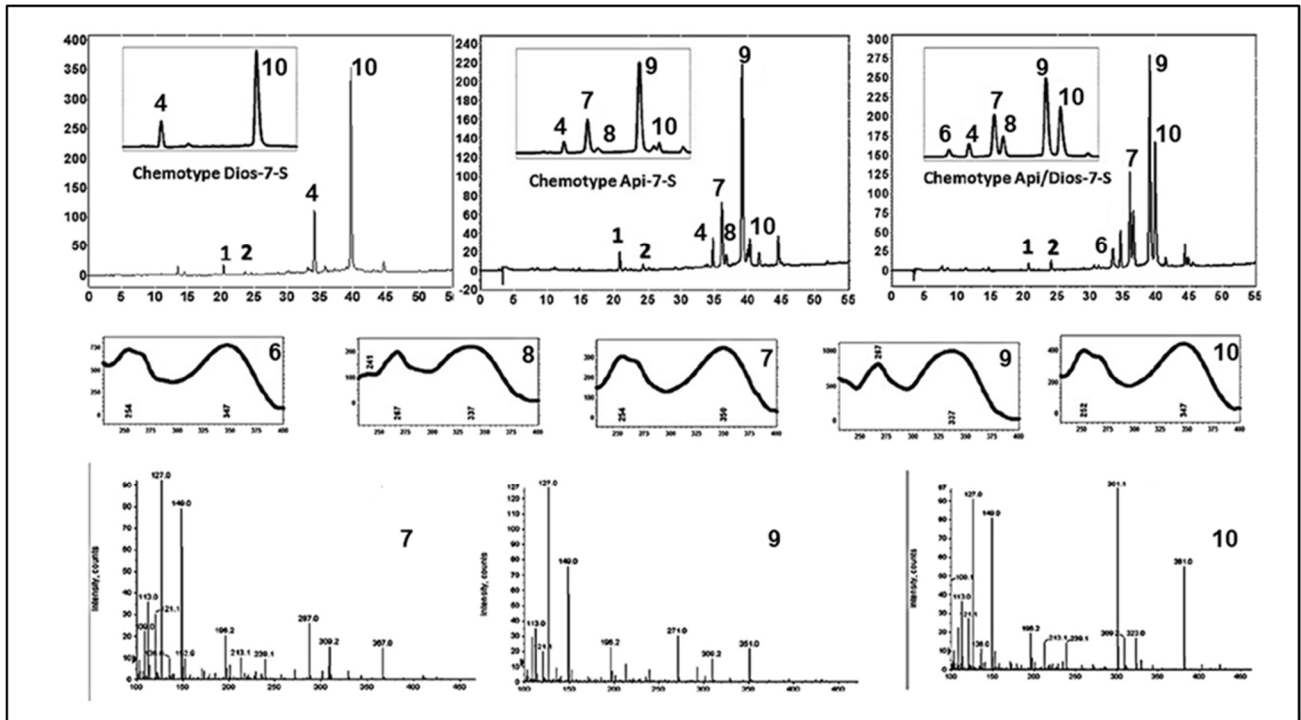


Figure S2. Top: HPLC profiles (280 nm) of the three flavonoid chemotypes of *N. noltei*; middle: online UV spectra of each flavonoid peak; bottom: mass spectra of flavonoid 7-sulfates 7, 9 and 10.

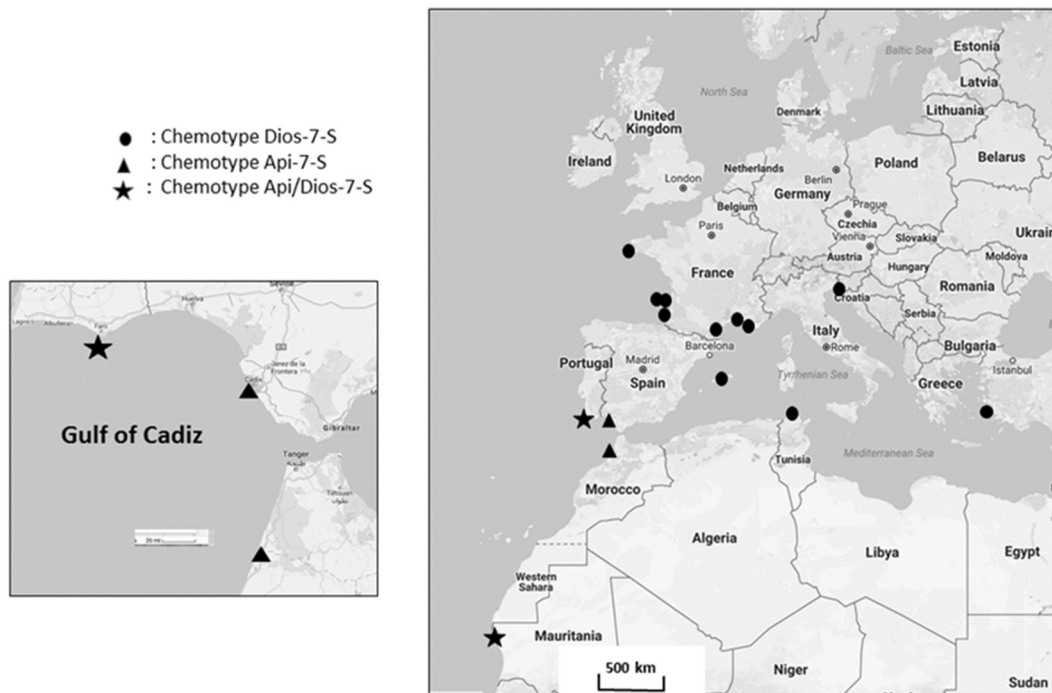


Figure S3. Map showing the geographic ranges of the flavonoid variants of *N. noltei*.

S3. Arcachon Bay, FR

Arcachon Bay (Fig. 1 and S3) is a 155 km² mesotidal system located on the south-western French Atlantic coast (44.58 N, -1.24 W). Tides are semidiurnal and enter the bay through two channels. The bay is connected to the Atlantic Ocean by a channel (2–3 km wide and 12 km long) that enables significant water exchanges. The inner bay is mostly composed of tidal flats, while the Arguin sandbank faces the wave-dominated shelf of the Bay of Biscay and is bordered by a large-scale tidal inlet. The amplitude of the semi-diurnal tide ranges from 1.1 to 4.9 m, with the large tidal mudflats (115 km²) being drained during low tide by the shallow tidal creeks in the inner part of the bay (156 km²). During high tide, surface water temperature and salinity fluctuations of 1–30 °C and 22–32 psu, respectively, occur annually. *N. noltei* beds are found on the intertidal mudflats between -0.3 m and +3.1 m above the level of the lowest tide. *Z. marina* is found in the subtidal area adjacent to the mudflats and on sandbanks (Fig. S4–5).

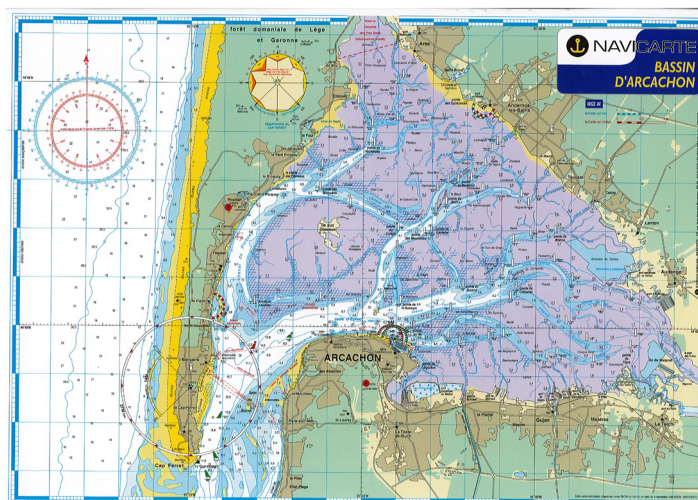


Figure S4. Morpho-bathymetric map of Arcachon Bay showing the underwater relief.

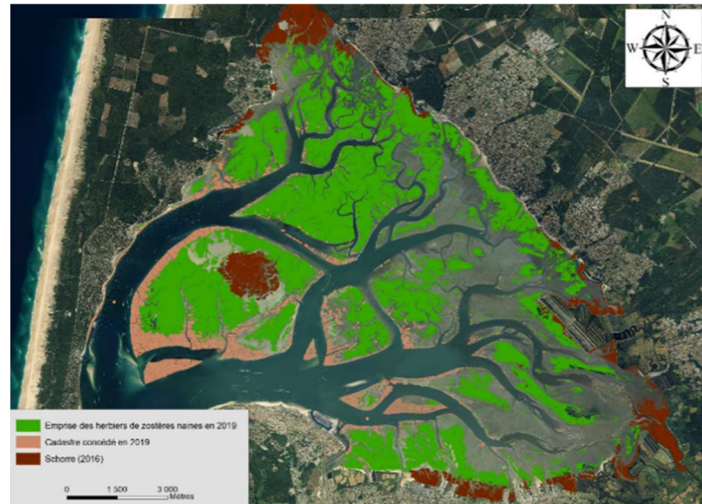


Figure 5. Mapping of *Zostera noltei* meadows in Arcachon Bay by hyperspectral imagery (Rigouin, L., Trut, G., Bajjouk, T., Rebeyrol, S., Liabot, P.O., Ganth, F., and Aubry, I. (2022)).

S4. Plant material

Specimens of *N. noltei* were collected in the inner part of the bay near Taussat (Figures S5 and S6). They are part of the Dios-7-S chemotype, found in the Mediterranean and the entire Atlantic coast of Europe, with the exception of the Ria Formosa and the Gulf of Cadiz (Figure 1 and S2).



Views of the surrounding *N. noltei* meadows from the study site



Figure S6. Location of study site off the Taussat coast: 44°42'56.02"N; 1°4'44.98" W; elevation 0.28 m.

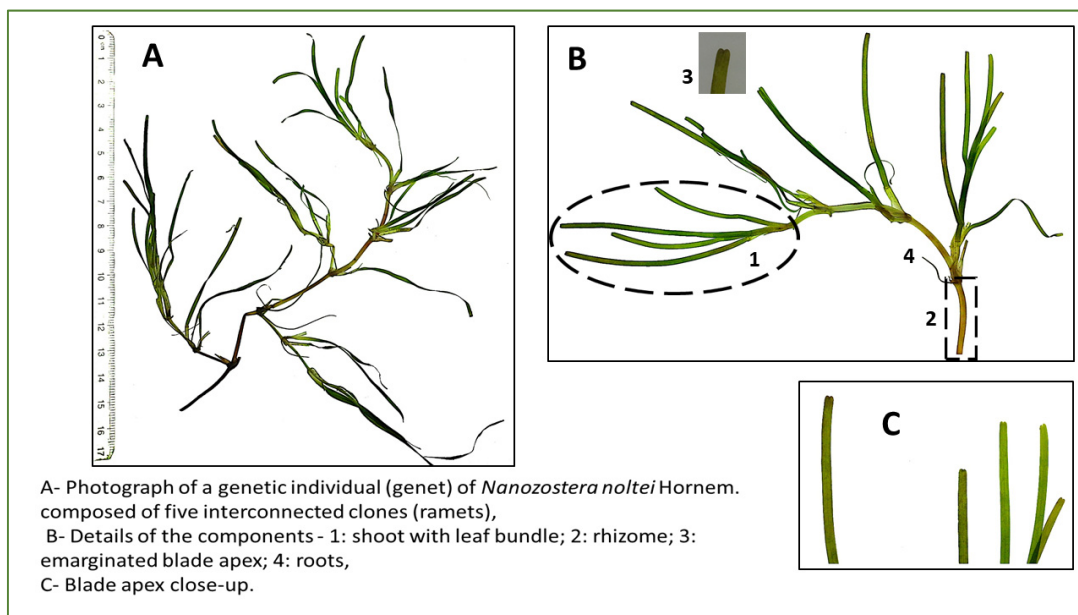
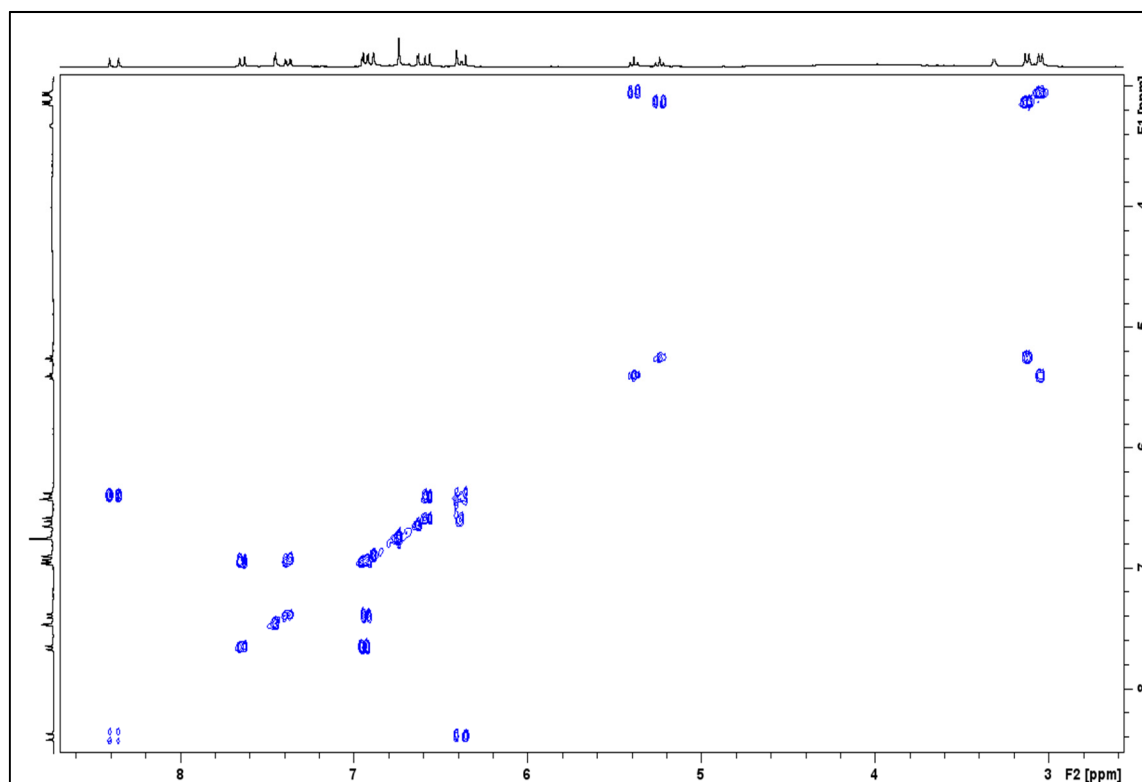


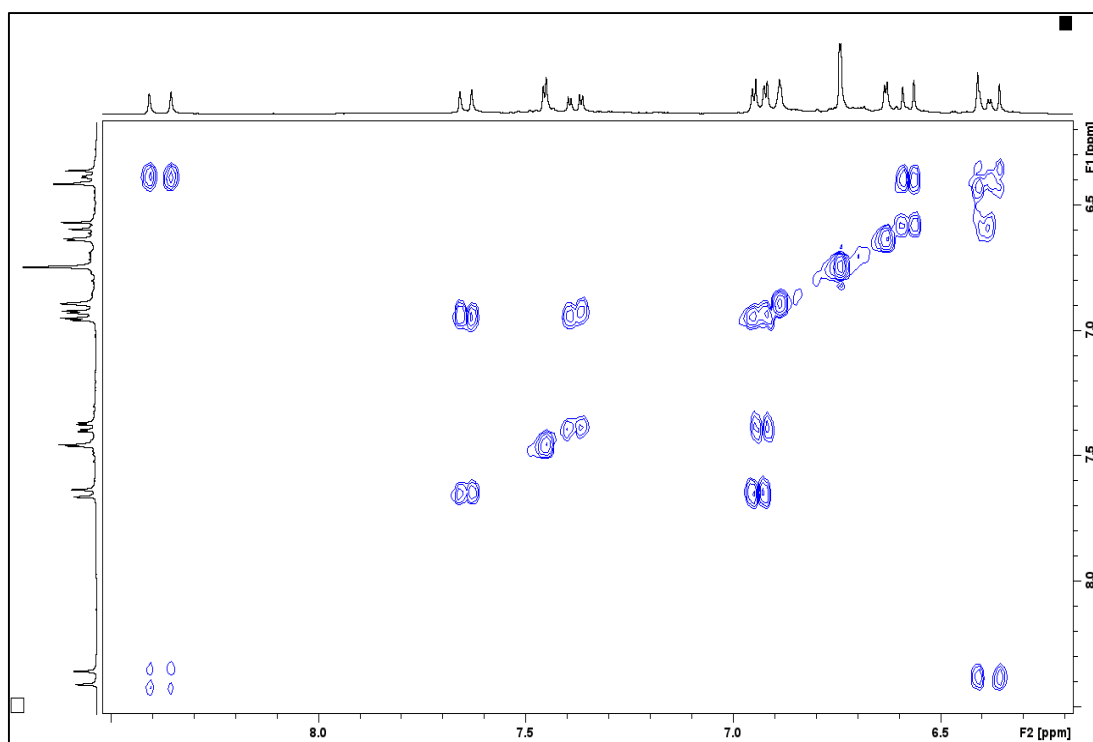
Figure S7. Photograph of a *N. noltei* sample from the study site (Arcachon Bay).

S5. Supplementary data for the structural analysis of zosteranoic acid (5)

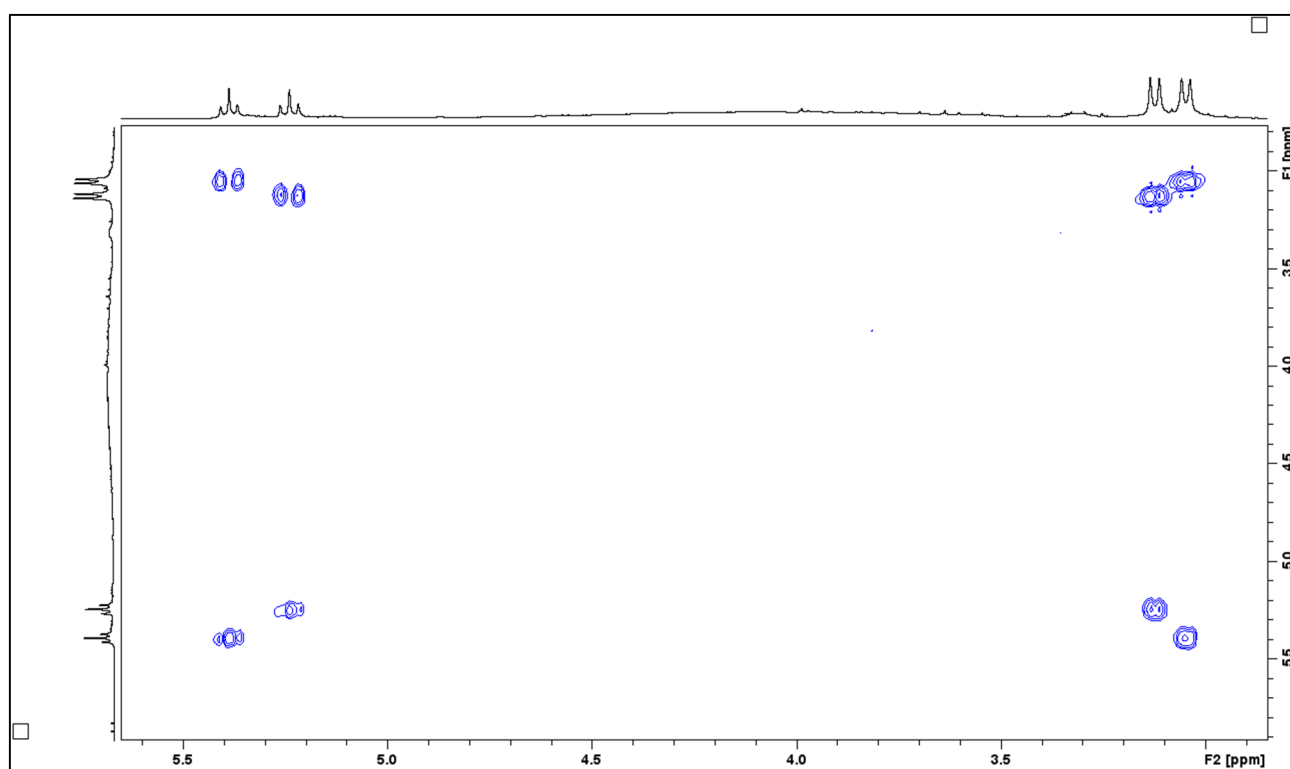
COSY



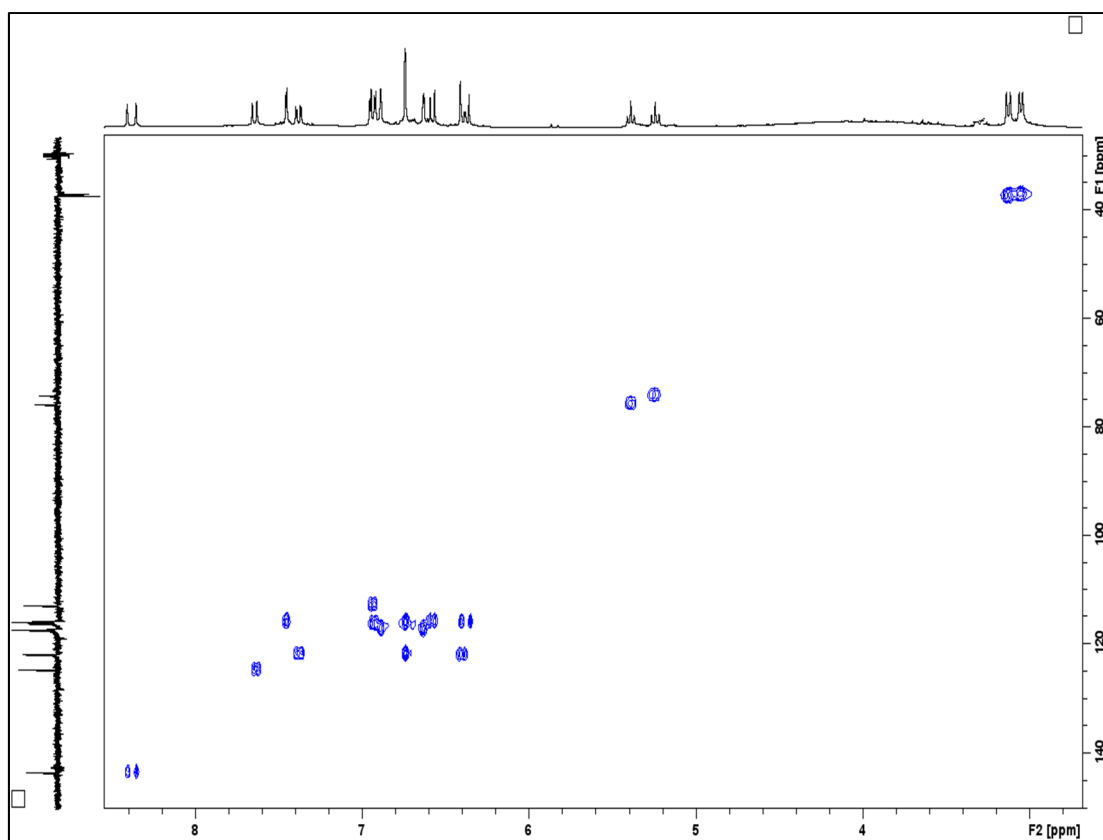
COSY: zoom on the aromatic region



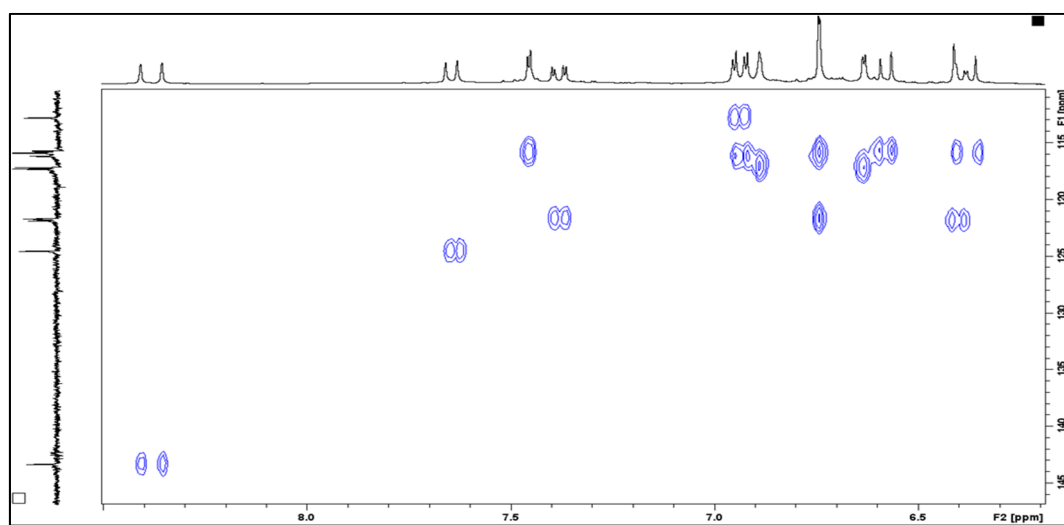
COSY: zoom on the aliphatic region



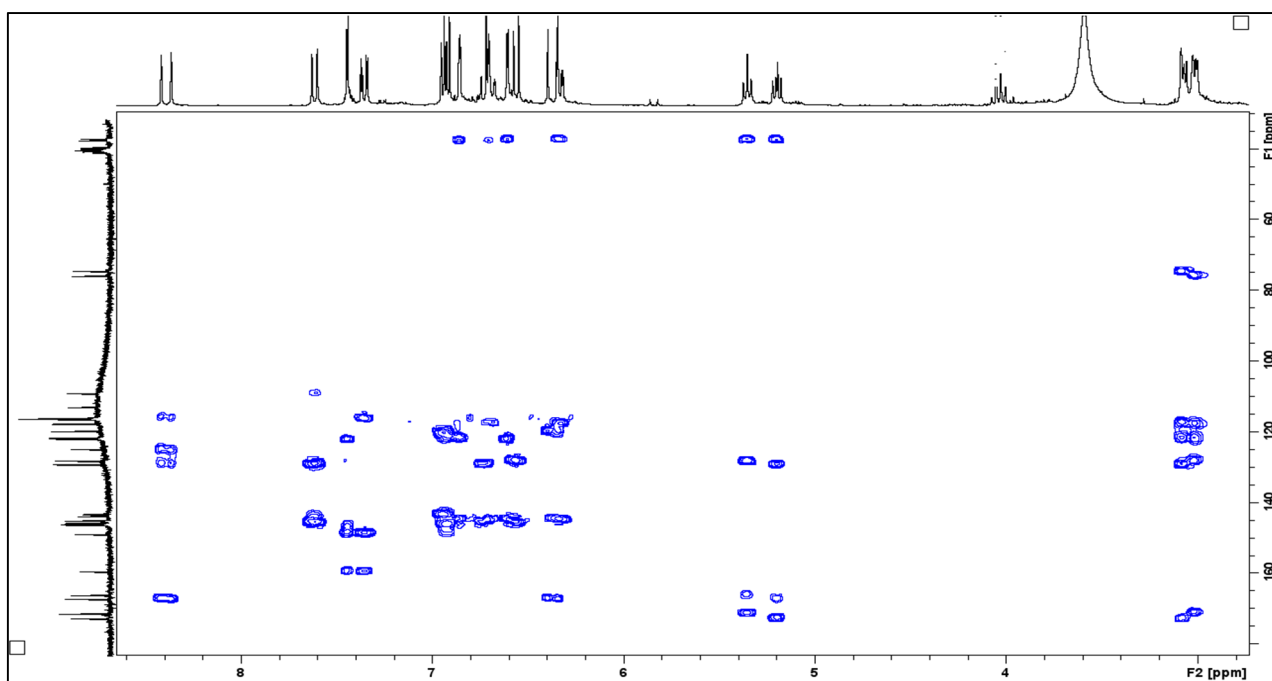
HSQC



HSQC: zoom on the aromatic region



HMBC



^{13}C

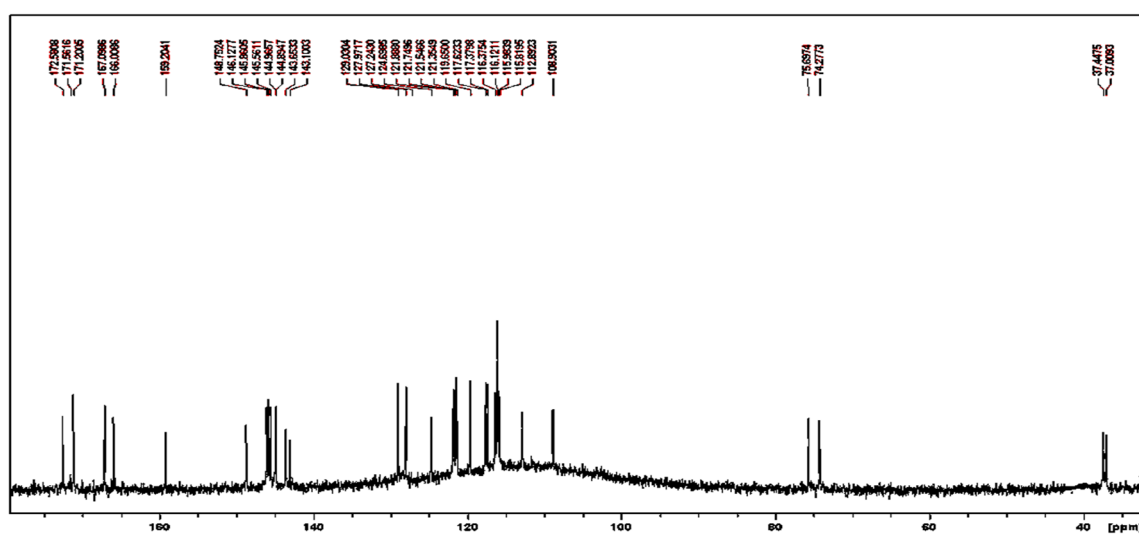


Figure S8. NMR spectra (300 MHz, Aceton- d_6).

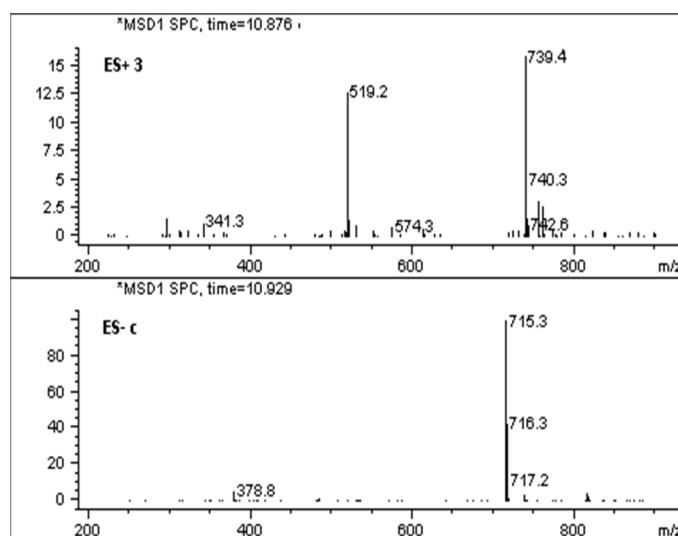


Figure S9. Mass spectra of compound **5** in positive and negative mode.

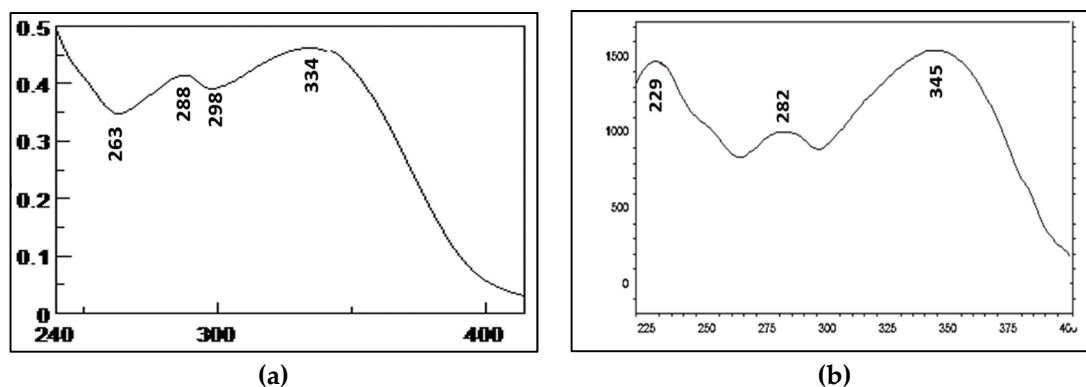


Figure S10. UV spectra of compound **5**: a) in methanol; b) UV-DAD online.

Table S1. ^1H and ^{13}C NMR data for compound **5** (Aceton- d_6).

Atom number	^1H (δ ppm, multiplicity, J Hz)	^{13}C (ppm)	Atom number	^1H (δ ppm, multiplicity, J Hz)	^{13}C (ppm)
1		120.1	1''		121.9
2		129.1	2''	7.45 (1H, d, 2.1)	115.9
3		143.1	3''		145.9
4		145.3	4''		148.5
5	6.94 (1H, d, 8.5)	112.9	5''	6.93 (1H, d, 8.3)	116.3
6	7.64 (1H, d, 6.9)	124.7	6''	7.38 (1H, dd, 8.3, 2.1)	121.8
7	8.38 (1H, d, 15.8)	143.5	7''		159.0
8	6.38 (1H, d, 15.8)	115.9	8''		109.0
9		167.0	9''		166.1
1'		129.2	1'''		128.1
2'	6.63 (1H, d, 2.01)	117.4	2'''	6.89 (1H, bs)	117.3
3'		145.4	3'''		145.7
4'		144.7	4'''		144.8
5'	6.58 (1H, d, 8.04)	116.0	5'''	6.74 (1H, d)	116.0
6'	6.39 (1H, dd, 8.1, 1.5)	121.6	6'''	6.74 (1H, m)	122.0
7'	3.12 (2H, d, 6.60)	37.4	7'''	3.05 (2H, d, 6.15)	37.1
8'	5.24 (1H, t, 6.60)	74.2	8'''	5.39 (1H, t, 6.15)	75.8
9'		172.7	9'''		170.1

Table S2. ¹H and ¹³C-NMR chemical shifts for flavonoid sulfates **7**, **9** and **10** (δ ppm, DMSO-*d*₆).

Atom number	Luteolin 7-sulfate (7)		Apigenin 7-sulfate (9)		Diosmetin 7-sulfate (10)	
	¹ H	¹³ C	¹ H	¹³ C	¹ H	¹³ C
2		164.5		164.7		163.9
3	6.74 [1 H, s]	103.2	6.94 [1 H, s]	103.4	6.83 [1 H, s]	103.4
4		182.1		182.8		182.2
5		160.6		161.2		160.5
6	6.51 [1 H, d (2.1)]	102.2	6.58 [1 H, d (2.0)]	105.1	6.54 [1 H, d (2.1)]	101.7
7		159.6		160.0		159.5
8	7.01 [1 H, d (2.1)]	97.7	7.02 [1 H, d (2.0)]	98.6	7.05 [1 H, d (2.1)]	97.3
9		156.4		160.0		156.3
10		105.3		106.5		105.7
1'		121.1		125.3		122.7
2'	7.44 [1 H, d (2.1)]	113.3	8.02 [1 H, d (8.8)]	129.5	7.48 [1 H, d (2.3)]	112.3
3'		145.1	7.35 [1 H, d (8.8)]	121.1		146.8
4'		150.3		156.8		151.1
5'	6.87 [1 H, d (8.9)]	116.2	7.35 [1 H, d (8.8)]	121.1	7.09 [1 H, d (8.6)]	111.9
6'	7.44 [1 H, dd (2.1, 8.9)]	119.3	8.02 [1 H, dd (8.8)]	129.5	7.58 [1 H, dd (2.3, 8.6)]	118.4
OMe		-		-	3.86 [3 H, s]	55.5

S6. Supplementary data for quantitative amounts of phenolic compounds

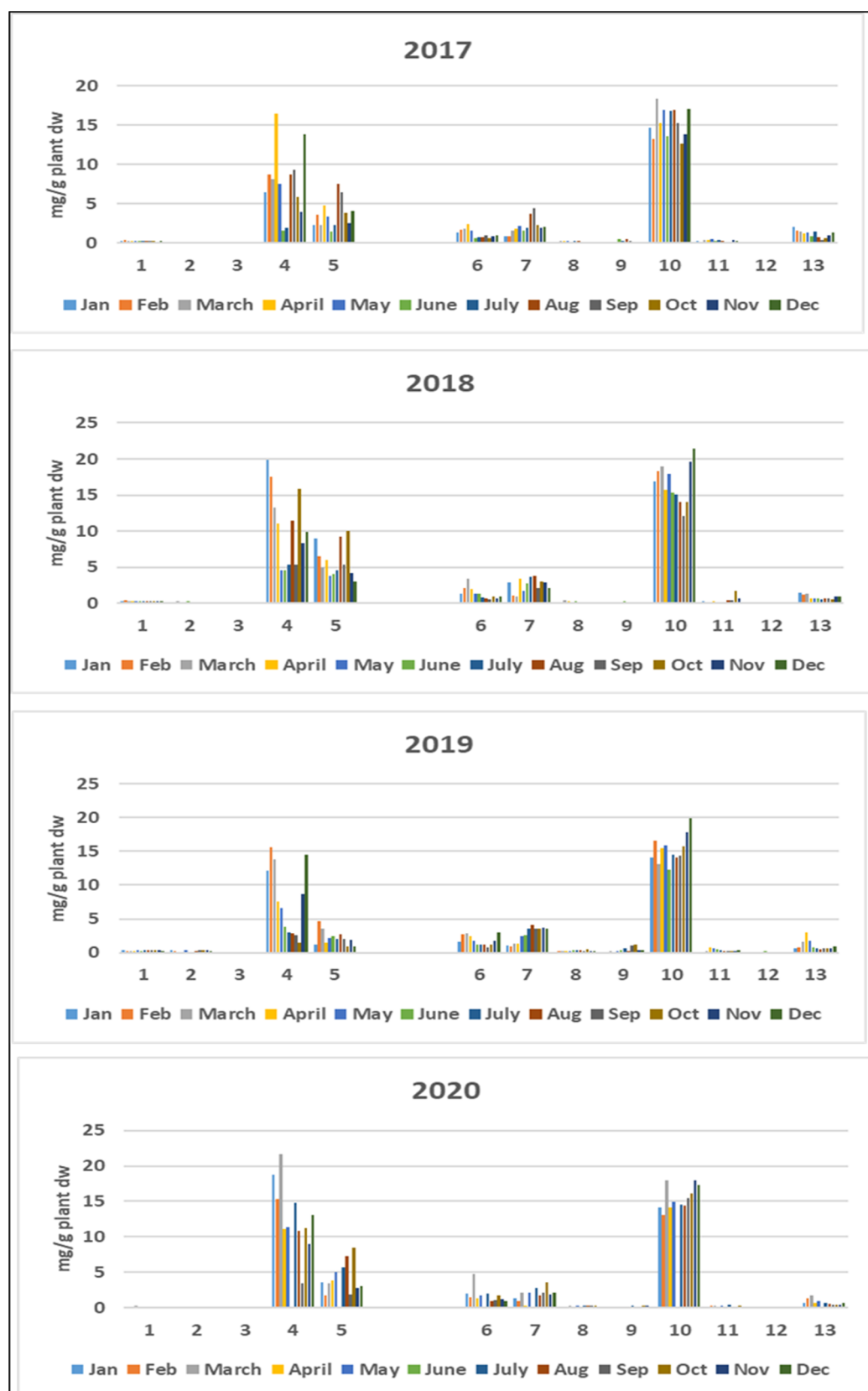


Figure S11. Histograms showing the variations of the quantitative amounts of phenolic compounds in *N. noltei* leaves over months and years. 1: zosteric acid; 2: caffeic acid; 3: coumaric acid; 4: rosmarinic acid; 5: zosteranoic acid; 6: luteolin 7-glucoside; 7: luteolin 7-sulfate; 8: apigenin 7-glucoside; 9: apigenin 7-sulfate; 10: diosmetin 7-sulfate; 11: luteolin; 12: apigenin; 13: diosmetin. Compounds are coded as in Tables 2–4 and Figure 2.

Table S3. Quantitative amounts (mg/g plant dried weight, mean values \pm SD) of individual phenolic compounds from specimen collected at other sites. A- *N. noltei* leaves from Atlantic and Mediterranean. B - *N. noltei* rhizomes from Hossegor, Thau and Ria Formosa. C- *Z. marina* rhizomes from Arguin, Hossegor, and Thau. Compounds are coded as in Tables 2-3 and Figures 2-3.

A												
Compound s	Arguin	Hossegor	Thau	Berres	Salses	Strunjan	Bizerte	Ria Formosa	Pointe de l'Etoile	Cadiz Bay	Merja Zerga	Concentrati on ranges
1	0.37±0.01	0.12±0.01	0.09±0.01	0.10±0.01	0.42±0.02	0.10±0.01	0.24±0.01	0.69±0.01	0.07±0.01	0.69±0.01	0.25±0.01	0.07-0.69
2	0.10±0.01	0.21±0.01	0.29±0.02	0.10±0.01	0.37±0.01	0.48±0.01	0.15±0.01	0.26±0.01	0.19±0.01	0.07±0.010	0.18±0.01	0.07-0.48
3	0	0	0	0	0	0	0	0	0	0	0	0
4	11.94±0.03	2.22±0.01	8.81±0.02	1.75±0.02	4.59±0.02	8.51±0.02	6.51±0.02	22.07±0.03	1.36±0.02	11.25±0.04	1.56±0.01	1.36-22.07
5	3.48±0.02	1.08±0.01	2.59±0.02	1.86±0.02	0.22±0.01	1.80±0.02	1.57±0.02	3.16±0.02	3.31±0.02	2.37±0.02	1.03±0.02	0.22-3.48
TPA	15.896	3.624	11.778	3.809	5.788	10.891	8.471	26.488	4.927	14.381	3.018	3.02-26.49
% of TP	41%	13%	36%	20%	26%	42%	31%	53%	21%	52%	15%	13-53%
6	0.63±0.01	1.42±0.02	0.71±0.02	0.06±0.01	0.48±0.01	0.35±0.01	0.06±0.01	0.68±0.01	0.66±0.01	0.17±0.01	0.12±0.01	0.05-1.42
7	1.83±0.01	1.44±0.02	0.79±0.02	0.42±0.01	1.19±0.02	0.55±0.01	0.58±0.02	5.76±0.02	3.84±0.02	5.76±0.02	3.92±0.02	0.42-5.76
8	0.06±0.01	0.17±0.01	0.05±0.01	0	0.07±0.01	0	0.17±0.01	0.88±0.02	1.66±0.02	0.69±0.02	0.51±0.01	0-1.66
9	0	0.30±0.02	0	0	0.09±0.01	0.04±0.01	0.19±0.01	7.65±0.02	6.75±0.02	3.18±0.02	9.56±0.02	0-7.65
10	19.84±0.02	20.96±0.03	18.31±0.03	13.20±0.02	13.78±0.03	20.86±0.04	17.17±0.03	6.68±0.02	5.06±0.02	0.64±0.01	0.78±0.02	0.64-20.96
11	0.29±0.02	0.24±0.02	0.11±0.01	0.10±0.01	0.25±0.02	0.06±0.01	0.11±0.01	0.69±0.02	0.30±0.02	0.51±0.01	0.71±0.02	0.06-0.71
12	0	0	0	0	0.080±0.009	0.084±0.009	0	0.643±0.014	0.562±0.013	1.773±0.018	1.227±0.017	0-1.773
13	0.59±0.02	0.66±0.02	0.84±0.02	1.48±0.02	0.28±0.02	0.22±0.02	0.76±0.02	0.78±0.02	0.28±0.01	0.42±0.01	0.12±0.01	0.12-1.48
TF	23.247	25.192	20.815	15.251	16.218	14.876	19.035	23.749	19.109	13.285	16.953	13.285-23.749
% of TP	59%	87%	64%	80%	74%	58%	69%	47%	79%	48%	85%	47-87%
TP	39.143	28.816	32.593	19.060	22.006	25.767	27.506	50.237	24.036	27.666	19.971	19.06-50.237
B				C								
Compound s	Ria Formosa	Hossegor	Thau	Concentratio n ranges	Arguin	Hossegor	Thau	Concentratio n ranges				
1	1.91±0.02	0.60±0.01	0.20±0.01	0.20-1.91	0.59±0.01	0.85±0.2	0.06±0.01	0.06-0.85				
2	0.08±0.01	0.05±0.01	0.08±0.01	0.05-0.08	0.12±0.01		0.13±0.01	0-0.13				
3		0.06±0.01	0.03±0.01	0-0.06	0.26±0.01	0.07±0.01	0.14±0.01	0.07-0.26				
4	3.55±0.02	1.38±0.02	2.27±0.02	1.38-3.55	3.93±0.02	3.25±0.02	4.54±0.02	3.25-4.54				
5	0.54±0.01	1.37±0.02	1.13±0.02	0.54-1.37	3.07±0.02	7.540.02	5.47±0.02	3.07-7.54				
TPA	6.08	3.46	3.71	3.46-6.08.	7.97	11.71	10.34	7.97-11.71				
% of TP	74%	73%	83%	73-83%	63%	90%	73%	63-90%				
6	0.13±0.01	0.07±0.01	0.28±0.01	0.07-0.28	0.41±0.01	0.57±0.01	1.43±0.02	0.41-1.43				
7	0.11±0.01	0.02±0.01		0-0.11	0.32±0.01	0.09±0.01	0.07±0.01	0.07-0.32				
8	0.20±0.01	0.01±0.01		0-0.20	0.05±0.01	0.09±0.01	0.12±0.01	0.05-0.12				
9	0.73±0.02	0.04±0.01		0-0.73	0.21±0.01	0.06±0.01	0.15±0.01	0.06-0.21				
10	0.72±0.02	0.77±0.02	0.33±0.01	0.33-0.77	1.30±0.02	0.19±0.01	0.51±0.01	0.19-1.30				
11	0.07±0.01	0.13±0.01	0.04±0.01	0.04-0.13	1.52±0.02	0.20±0.01	1.28±0.02	0.20-1.52				
12	0.12±0.01			0-0.12	0.44±0.02		0.18±0.02	0-0.44				
13	0.11±0.01	0.24±0.01	0.10±0.01	0-0.24	0.49±0.02	0.08±0.01	0.18±0.01	0.08-0.49				
TF	2.19	1.28	0.75	0.75-2.19	4.74	1.28	3.92	1.28-4.74				
% of TP	26%	27%	17%	17-26%	37%	10%	27%	10-37%				
TP	8.27	4.74	4.46	4.46-8.27	12.71	12.99	14.26	12.71-14.26				

S7. Supplementary data for Principal Component Analyses and Hierarchical Cluster Analysis

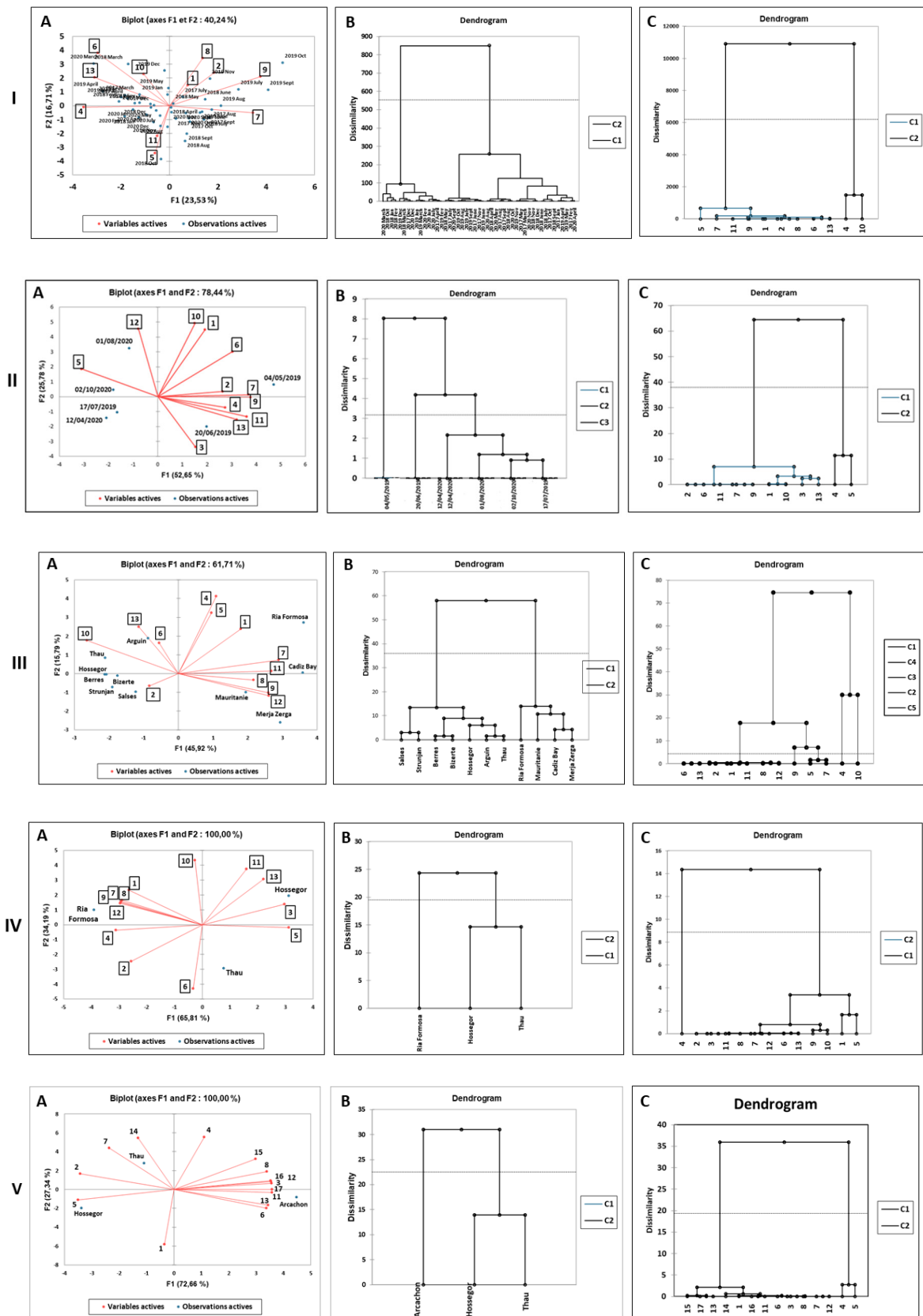


Figure S12. Principal component and hierarchical cluster analysis for: I— *N. noltei* leaves from Arcachon Bay; II— *N. noltei* rhizomes from Arcachon Bay; III— *N. noltei* leaves from other sites; IV— *N. noltei* rhizomes from other sites; V— *Z. marina* rhizomes from other sites. In every case: A—PCA biplot graph based on PC1 and PC2 scores; B—dendrogram based on the phenolic composition; C—dendrogram obtained from the cluster analysis of the variables. Compound numbers, collection dates and locations are the same as in Tables 1–4 and Figures 2–3.

S7.1. N. noltei leaves from other sites in the Atlantic and Mediterranean

Eleven populations representative of the three geographically distinct flavonoid chemotypes were considered (Figures 1 and S2, Table 1). The relationships between the populations (observations) and the phenolic compound concentrations determined by HPLC (variables) are depicted in Figure 6 III-A. The PCAs showed a cumulative variance of 61.71% up to the second principal component with values of F1 and F2 equal to 45.92% and 15.79%, respectively (Figure S12 III-A). The first principal component is strongly correlated with flavonoids 7-9 and 11-12 (positive associations) and with flavonoid 10 (negative association). The second principal component increases with rosmarinic acid (4) and zosteranoic acid (5), which are strongly correlated to each other. The PCA biplot graphic F1 x F2 clearly distinguishes the three geographical flavonoid chemotypes (Figures 1 and S2), with Merja Zerga and Cadiz Bay (chemotype Api-7-S) and Ria Formosa and Mauritania (chemotype Api/Dios-7-S) positively correlated to F1 and flavonoids 7 and 9, while all the other locations (chemotype Dios-7-S) negatively correlated to F1 and diosmetin 7-sulfate (7).

The dendrogram obtained from the cluster analysis of the eleven populations in relation to the concentrations of the chemical constituents is presented in Figure S12 III-B. The automatic truncation identified two clusters (C1 and C2), which occur at a dissimilarity level of approximately 36. Cluster C1 comprises the eleven populations representative of the chemotype Dios-7-S. Cluster C2 gathers the populations representative of chemotypes Api-7-S and Api/Dios-7-S, which are separated at a dissimilarity level of approximately ten.

The dendrogram obtained from the cluster analysis of the variables identified five clusters, respectively constituted of compounds 1-2, 6, 8 and 11-13 (C1); 9 (C2); 5 and 7 (C3); 4 (C4) and 10 (C5) (Figure S12 III-C).

S7.2. N. noltei rhizomes from Hossegor Lake, Ria Formosa and Thau Lagoon

The relationships between the three populations (observations) and the phenolic compound concentrations determined by HPLC (variables) are depicted in Figure S12 IV-A. It shows a cumulative variance of 100% up to the second principal component with values of F1 and F2 equal to 65.81% and 34.19%, respectively. The first principal component is strongly correlated with coumaric acid (3) and zosteranoic acid (5) (positive associations) and with rosmarinic acid (4) and flavonoids 7-9 and 12 (negative associations). The second component has large positive associations with flavonoids 10 and 13 and large negative associations with flavonoid 6 and with zosteric acid (1). F1 is positively correlated to Hossegor and negatively to Ria Formosa, while Thau is negatively correlated to F2.

The dendrogram obtained from the cluster analysis of the three locations in relation to the concentrations of chemical constituents is presented in Figure S12 IV-B. The automatic truncation identified two clusters (C1, C2), which occur at a dissimilarity level of approximately 19. Cluster C1 includes Ria Formosa, and C2 gathers Hossegor Lake and Thau Lagoon.

The dendrogram obtained from the cluster analysis of the variables also identifies two clusters at a dissimilarity level of approximately nine (Figure S12 IV-C). Cluster C1 only contains rosmarinic acid (4), while C2 groups together all the other compounds

3.3.4. Z. marina rhizomes from Hossegor Lake, Arcachon Bay and Thau Lagoon

The relationships between the three populations (observations) and the phenolic compound concentrations determined by HPLC (variables) are depicted in Figure 12 V-A. It shows a cumulative variance of 100% up to the second principal component with values of F1 and F2 equal to 72.66% and 27.34%, respectively. The first principal component is strongly correlated with coumaric acid (3) and eight of the ten flavonoids (positive associations) and with caffeic (2) and zosteranoic acids (5) (negative associations). The second component has a large positive association with rosmarinic acid

(4) and a large negative association with zosteric acid (1). F1 is positively correlated to Arcachon Bay, and negatively to Hossegor Lake and Thau Lagoon.

The dendrogram obtained from the cluster analysis of the three locations in relation to the concentrations of chemical constituents is presented in Figure 12 V-B. The automatic truncation identified two clusters (C1, C2), which occur at a dissimilarity level of approximately 22. Cluster C1 includes Arcachon Bay and C2 gathers Hossegor Lake and Thau Lagoon.

The dendrogram obtained from the cluster analysis of the variables also identifies two clusters at a dissimilarity level of approximately 20 (Figure 12 V-C). Cluster C1 contains all the compounds except rosmarinic acid and zosteranoic acid, which constitute C2.

S8. Supplementary data for HPLC

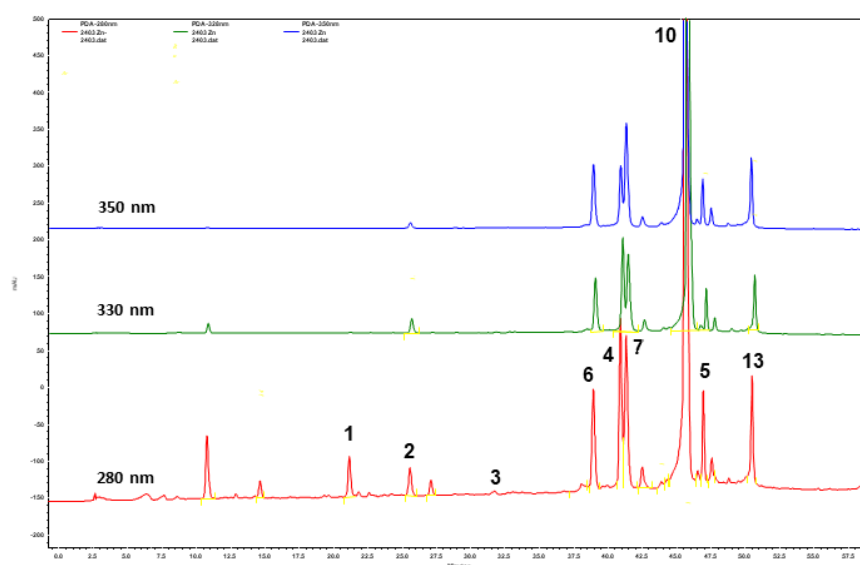


Figure S13. HPLC profile of *Nanozostera noltei* crude extract from leaves recorded at 280, 330 and 350 nm. Peak number assignment—1: zosteric acid; 2: caffeic acid; 3: coumaric acid; 4: rosmarinic acid; 5: zosteranoic acid; 6: luteolin 7-glucoside; 7: luteolin 7-sulfate; 10: diosmetin 7-sulfate; 13: diosmetin. Compounds are coded as in Tables 2–5 and Figure 2.