Supporting information

Nanobody-Based Enzyme Immunoassay for Naringenin in Pummelos and Herbal Samples

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Buffers:

(1) The phosphate-buffered saline (PBS, 10X, 0.1M) was supplied by Sangon Biotech Co., Ltd (Shanghai, China).

(2) Coating buffer (CBS): 0.05 M sodium carbonate buffer with pH of 9.6.

(3) Blocking buffer: 0.01 M PBS containing 3% non-fat powdered milk.

(4) Washing buffer (PBST): 0.01 M PBS containing 0.1% Tween-20.

(5) Sample dilution buffer (PBSTG): 0.01 M PBS containing 0.1% Tween-20 and 0.5% gelatin.

(6) Citrate buffer: 23.02 g of potassium dihydrogen citrate and 0.05 g of potassium sorbate, dissolved in deionized water and set to 500 mL.

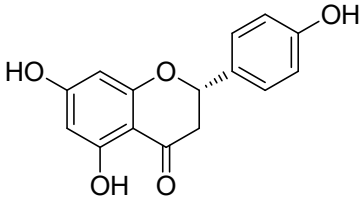
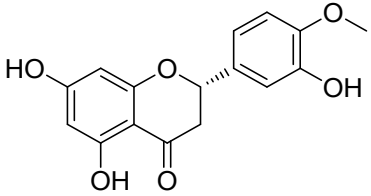
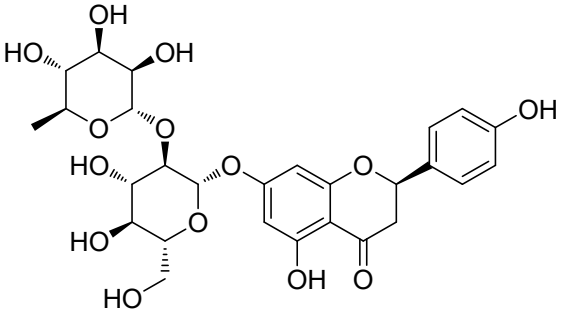
(7) TMB substrate solution: 11 mL of citrate buffer, 101 μL of 1% H_2O_2 and 200 μL of 0.6% TMB in DMSO.

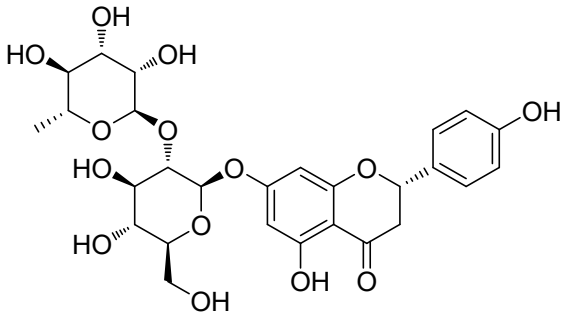
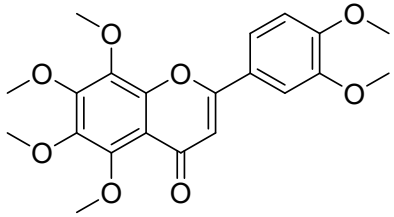
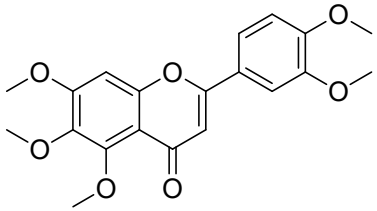
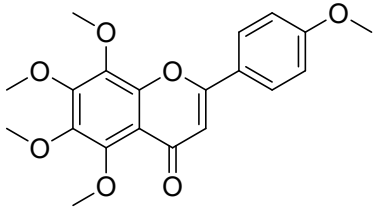
(8) Stop buffer: 2.0 M H_2SO_4.

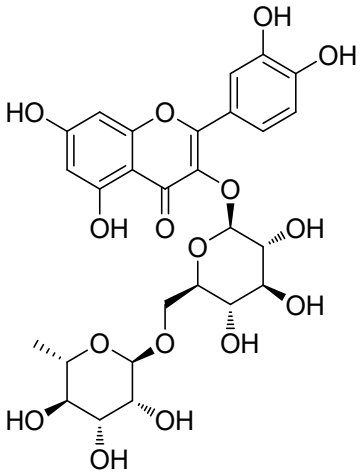
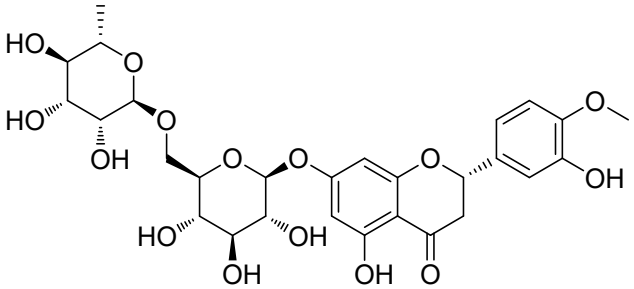
32 **Table S1. Sequences of the primers.**

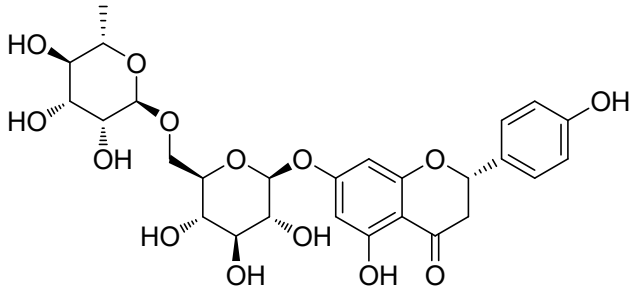
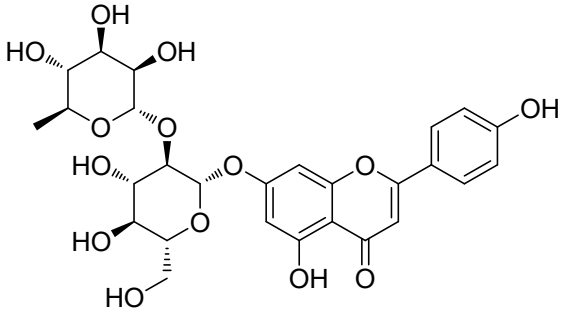
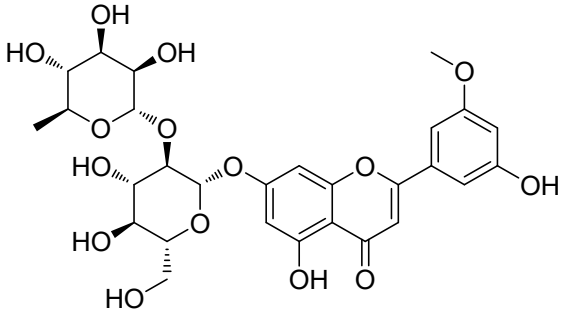
Primers	Sequences
Alp-Vh-F1	CATGCCATGACTGTGGCCCAGGCGGCCCAGKTGCAGCT CGTGGAGTC
AlpVhh-R1	CATGCCATGACTCGCGGCCGGCCTGGCCATGGGGGTCTT CGCTGTGGTGCG
AlpVhh-R2	CATGCCATGACTCGCGGCCGGCCTGGCCGTCTTGTGGTT TTGGTGTCTTGGG
Pseq	TTCCGGCTCGTATGTTGTGTG

Table S2. Cross-reactivity of the antibodies against Naringenin structural analogs.

Flavonoid	Structure	C9		C13	
		IC_{50} (ng/ml)	CR(%)	IC_{50} (ng/ml)	CR(%)
Naringenin		15.31	100.00	19.60	100.00
Hesperitin		4103.00	0.37	3970.00	0.49
Poncirin		-	<0.1	-	<0.1

Naringin		-	<0.1	-	<0.1
Nobiletin		-	<0.1	-	<0.1
Sinensetin		-	<0.1	-	<0.1
Tangeretin		-	<0.1	-	<0.1

Rutin		-	<0.1	-	<0.1
Hesperidin		-	<0.1	-	<0.1

Narirutin		-	<0.1	-	<0.1
Rhoifolin		-	<0.1	-	<0.1
Neohesperidin		-	<0.1	-	<0.1

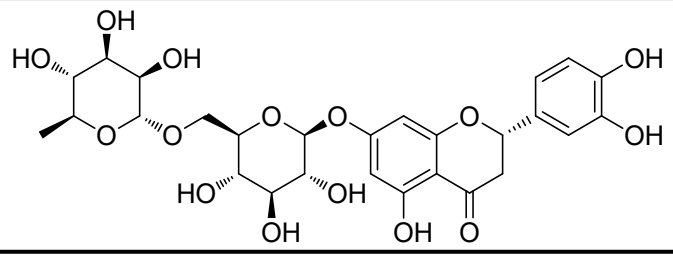
Eriocitrin		-	<0.1	-	<0.1

Table S3. Comparison of analytical performance and characteristics of the developed Nb-ELISA with other reported methods for naringenin detection.

Method	Key analytical figures	Pros	Cons	Reference
Nb-ELISA	IC_{50} = 15.31 ng/mL; Linear range: 4.55–60.60 ng/mL	High thermal stability; nanobody-based recognition; simple operation; cost-effective; suitable for high-throughput screening; no need for expensive instrumentation	Moderate sensitivity compared to instrumental methods; requires specific nanobody production	this study
icELISA (conventional monoclonal antibody)	IC_{50} = 4.43 ng/mL; Linear range: 1.15–15.81 ng/mL	Reliable and accurate; established immunoassay format	Lower thermal stability than nanobodies; narrower linear range; antibody production more complex	[26]
GC-FID / GC-MS	LOD: 1.13–2.72 μg/g (in leaves); RSD: 1.65–1.81%	Rapid; good repeatability; suitable for volatile compounds	Requires derivatization; high instrument cost; not ideal for non-volatile naringenin; sample preparation intensive	[27]
Chromatographic method (HPLC/UPLC)	RSD < 0.9%; Accuracy > 98%	Fast; sensitive; gold standard for quantification	High instrument and maintenance cost; time-consuming for large sample sets; requires skilled personnel	[28]
Electrochemical sensor	Linear range: 1.0×10^{-13}–1.0×10^{-12} mol/L; LOD: 2.84×10^{-14} mol/L; LOQ: 9.47×10^{-14} mol/L	Cost-effective; high efficiency; extremely low LOD; suitable for standard solutions	Limited to standard solutions; matrix effects in real samples; poor stability in complex biological/plant matrices	[30]

Fluorescence method	Linear range: 0–200 μmol/L; LOD: 0.33–0.83 μmol/L	Simple; rapid; high efficiency	Relatively low sensitivity; interference from autofluorescence in plant extracts	[31]
MEKC	Linear range: 0.1–50 μg/mL; LOD: 0.05 μg/mL; LOQ: 0.19 μg/mL; $R^2 = 0.995$	Good resolution; low sample consumption; moderate sensitivity	Requires capillary electrophoresis system; not high-throughput; method development can be complex	[29]

Fig S1. Thermostability of naringenin-specific nanobodies Nb-C9 (■), Nb-C13 (●) and mAb-2A3D7 (▲). (A) Nanobodies and conventional monoclonal antibody were diluted to working concentrations in PBS and were incubated at 85 °C for various times, 0, 5, 15, 25, 35, 45 and 60 min; (B)Nanobodies and conventional monoclonal antibody were diluted to working concentrations in PBSTG and were heated at various temperatures, 20, 50, 65, 75 and 95 °C for 5 min.