

**THE APPLICATION OF UHPLC/HRMS TO THE DETERMINATION OF  
COUMARIN AND P-COUMARIC ACID IN HONEY SAMPLES**  
**THEME OF CZECH CONTRIBUTION:** Stanovení kumarinů v medové matrici  
v **problematice posuzování míry rizika toxicity medu**

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# HONEY, ELEMENTAR FACTS

- Honey is produced naturally by honeybees in their hives.
- It's made from nectar that the bees collect from flowers and water.
- The water is then evaporated and the golden liquid we all know and love as honey, is produced.
- The other constituents of honey are amino acids, antibiotic-rich inhibine, proteins, **phenol antioxidants**, and micronutrients

# Phytochemical Background of Coumarins

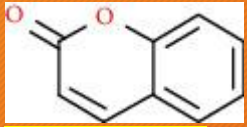
- Benzopyrene family
- Many medicinal plants:
- Coumarouna odorata (tonka bean), Fabaceae/Leguminosae
- Anthoxanthum odoratum( vanilla grass)
- Gen.Melilotus (sweet clover)
- Cassia cinnamon(Cinnamomum cassia)
- Cherry blossom trees (gen.Prunus)
- Apiaceae

## Czech List´s Botanical Names of Coumarine-Content Plant Commonly Occured in Czech Kitchens

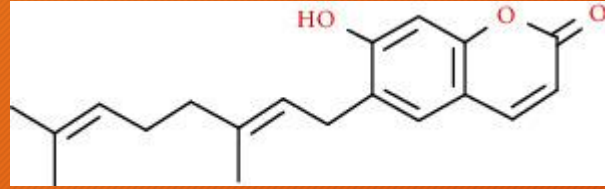
- andělíka lékařská (*Archangelica officinalis*)
- blín černý (*Hyoscyamus niger*)
- citronečník trojlistý (*Poncirus trifoliata*)
- divizna (*Verbascum*)
- durman obecný (*Datura stramonium*)
- heřmánek pravý (*Matricaria chamomilla*)<sup>!</sup>
- jitrocel kopinatý (*Plantago lanceolata*)
- kalina obecná (*Viburnum opulus*)
- komonice bílá (*Melilotus albus*)
- komonice lékařská (*Melilotus officinalis*) - cca 0,9 % of coumarine in blossoms, as aglycone, evaporates during dry processing
- komonice nejvyšší (*Melilotus altissimus*)
- levandule lékařská (*Lavandula angustifolia*)
- mařinka vonná, or svízel vonný (*Galium odoratum*)
- pelyněk (*Artemisia*)
- pískavice řecké seno (*Trigonella foenum-graecum*)
- routa vonná (*Ruta graveolens*) -photosensitizing furocoumarines,a risk of skin inflammation
- skořicovník ceylonský (*Cinnamomum zeylanicum*) - bark, cz. skořice
- skořicovník čínský (*Cinnamomum cassia*) - bark, basically stronger then Cinna descript above
- silvoň obecný (*Dipteryx odorata*)-beans tonka cz tonkové boby
- tomka vonná (*Anthoxanthum odoratum*)<sup>!</sup>
- tomkovice vonná (*Hierochloë odorata*) - vodka Zubrowka (Poland)
- vanilka pravá, or vanilovník plocholistý (*Vanilla planifolia*)
- vstavač nachový (*Orchis purpurea*)

# Natural Coumarins - Link between their Chemical Structures and Pharmacological Effects

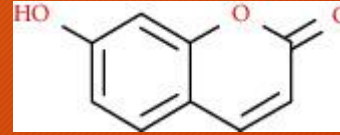
- 2H-1-benzopyran-2-one
- The term coumarin derives from *Coumarona odorata* (S.America), c.first isolated in the **1820s**
- Original source of coumarins: *Dipteryx odorata* Wild (tonka bean)
- **150** plants from families: Apiaceae/  
Umbelliferae, Caprifoliaceae, Clusiaceae,  
Guttiferae, Nyctaginaceae, Oleaceae, Rutaceae



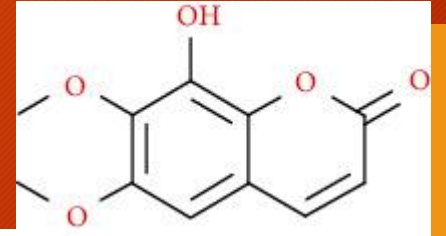
Coumarin



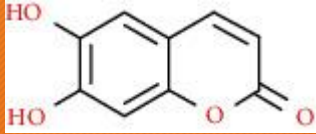
Ostruthin



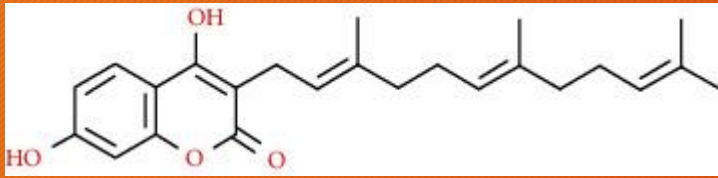
Umbelliferone



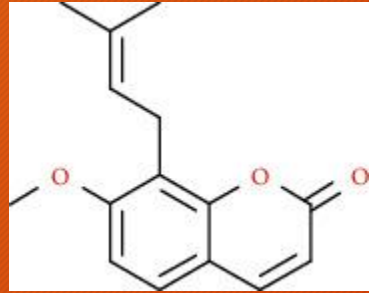
Fraxidin



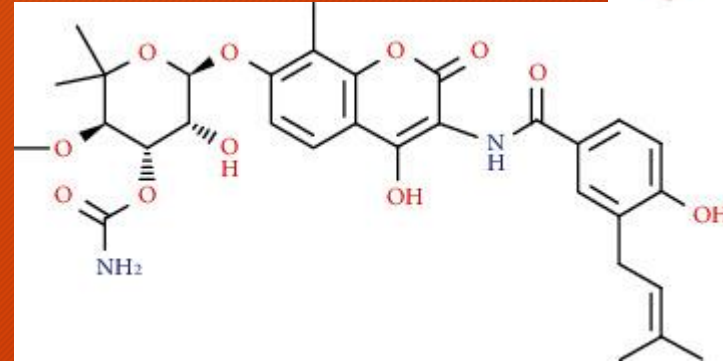
Esculetin



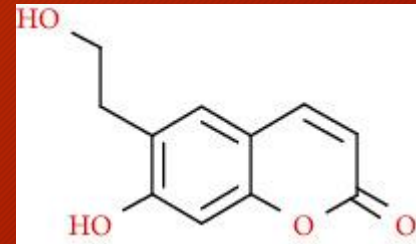
Ammoresinol



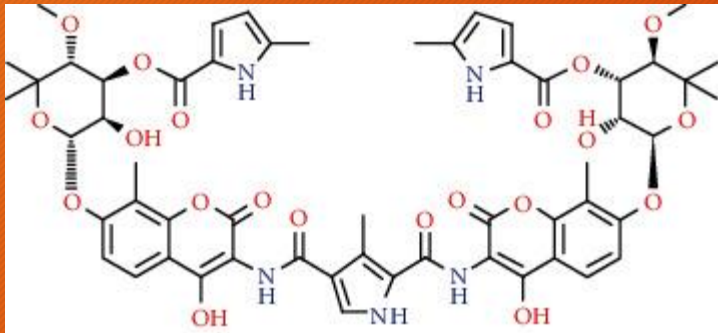
Osthole



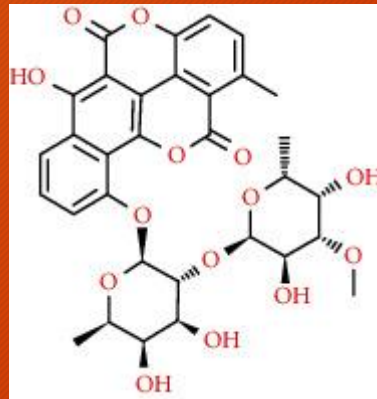
Novobiocin



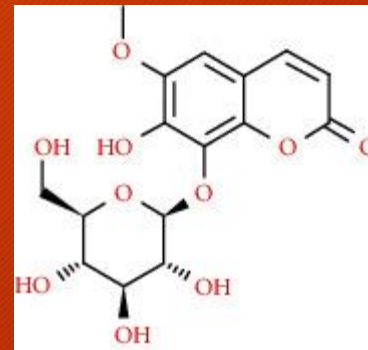
Phellodenol A



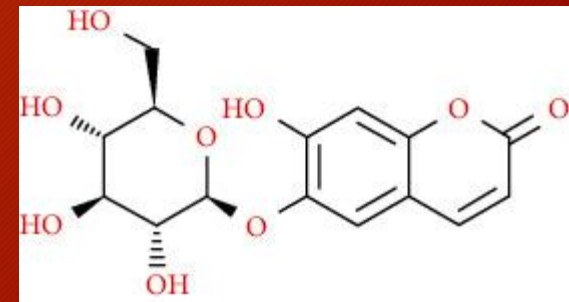
Coumermycin



Chartreusin

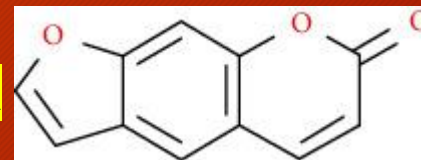


Fraxin



Esculin

Psoralen



# Naturally occurring simple coumarins

- Coumarin anti-inflammatory
- Esculetin anti-inflammatory, anticancer, neuroprotective
- Ammosesinol antibacterial
- Ostruthin antibacterial
- Osthole antioxidant, antimicrobial, antitumor, anticonvulsant, antidiabetic, antibacterial
- Novobiocin antibacterial
- Coumermycin antibacterial
- Chartreusin antibacterial, anticancer
- Fraxin antioxidant, anticancer, antiadipogenic
- Umbelliferone antitubercular
- Fraxidin antiadipogenic, antihyperglycemic
- Phellodenol A antitubercular
- Esculin antiadipogenic

# Naturally occurring dihydrofurano coumarins

- Anthogenol antibacterial, antituberculosis
- Felamidin
- marmesin



# Naturally occurring furano coumarins

- Imperatorin            antiinflammatory, antimicrobial, anticancer, anticonvulsant
- Psolaren            antifungal, antituberculosis
- Bergapten            antituberculosis
- Methoxsalen            cytochrome P450 inhibitor

# Naturally occurring linear coumarins

- Agasylin     antibacterial
- Aegelinol   antibacterial
- Xanthyletin antituberculosis

# Naturally occurring angular coumarins

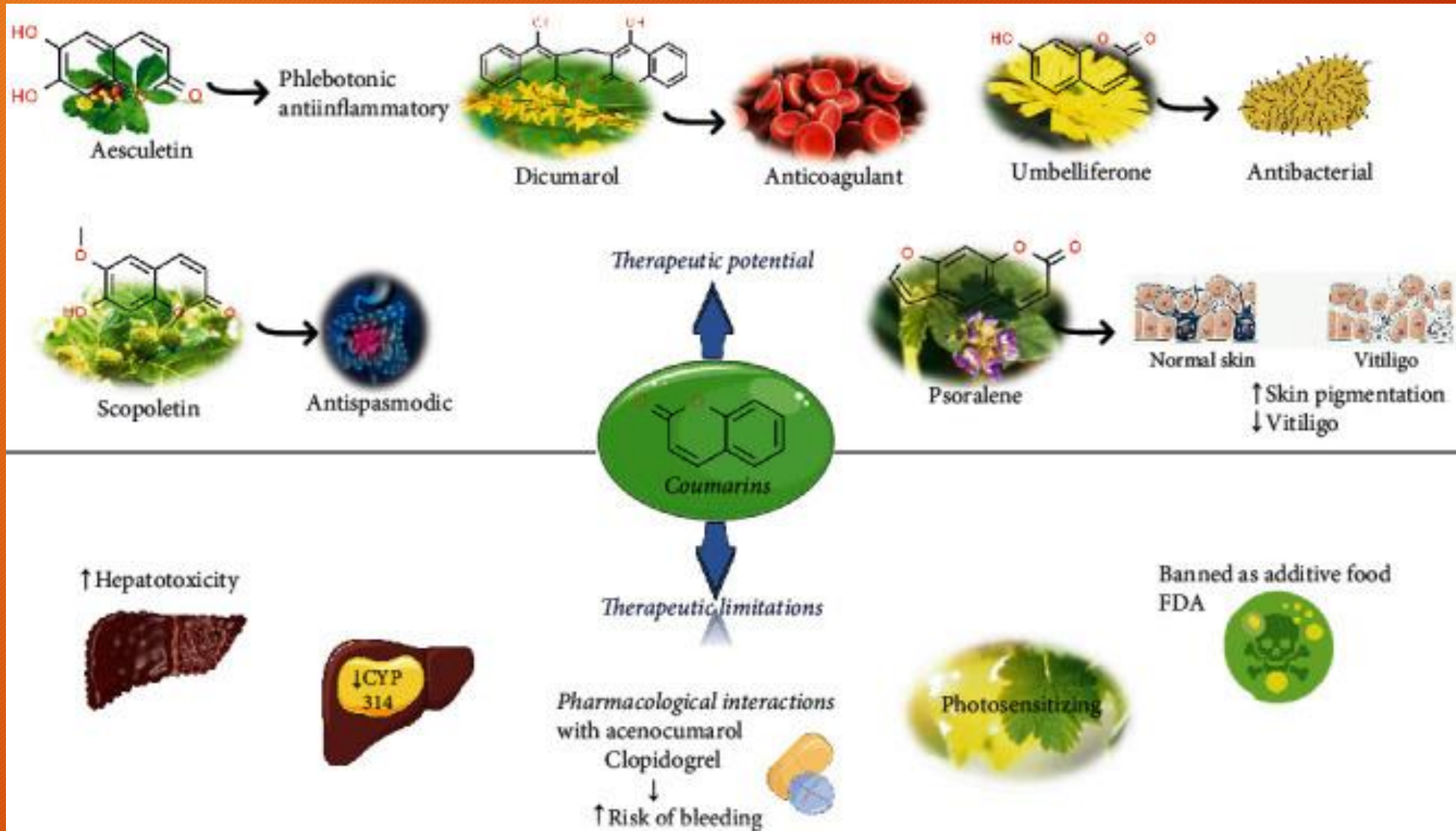
- Inophyllum A,B,C,E,P,G1, G2     antiviral
- Calanolide A, B,F
- Dihydrocalanolide A,B
- Pseudocordatolide C

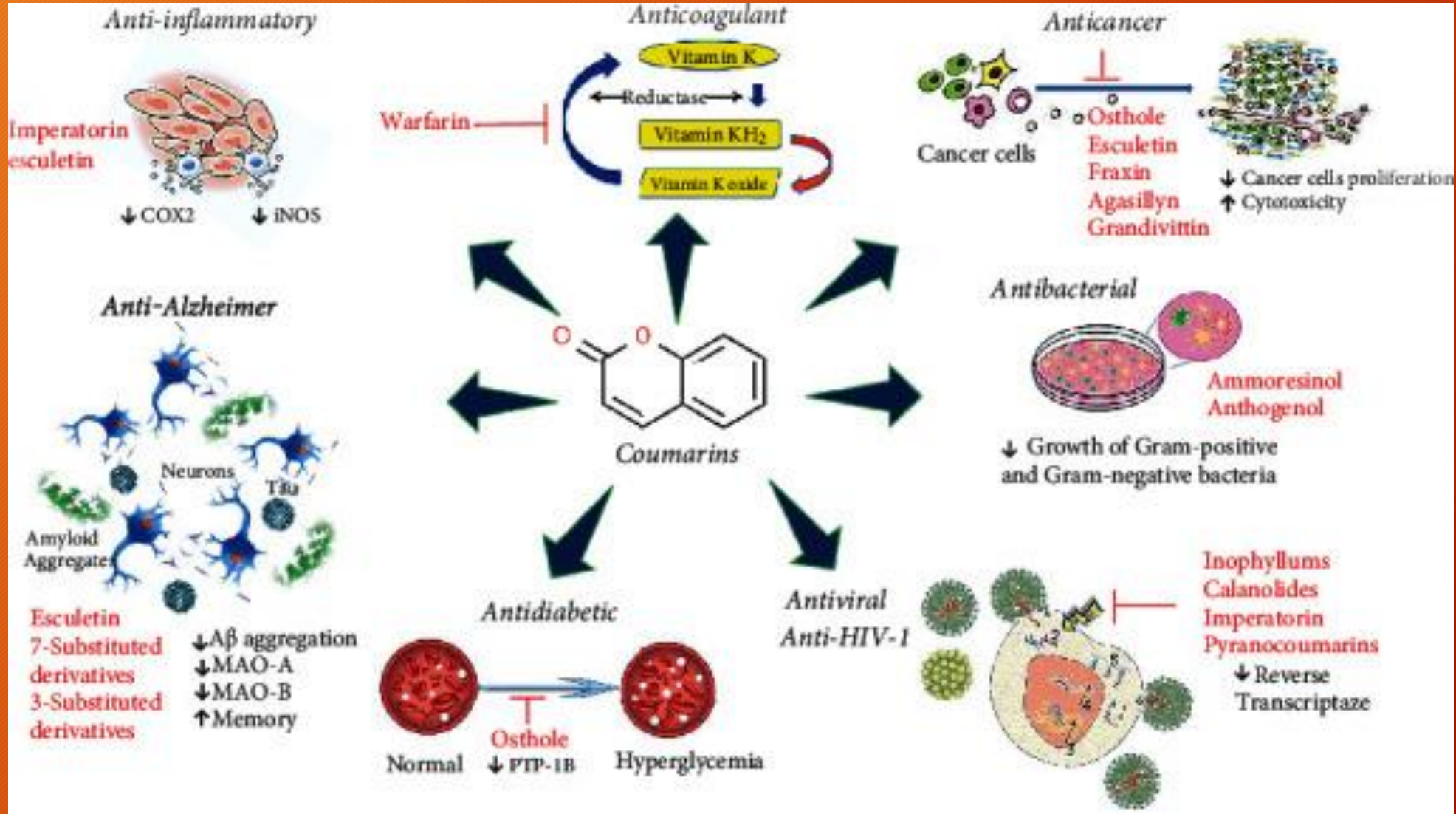
# Naturally occurring bi coumarins

- Dicumarol anticoagulant

# Pharmacology activities of coumarins

- Anti-inflammatory
- Anticoagulant
- Anticancer
- Antibacterial
- Antimalarial
- Casein kinase-2(CK2)
- Antifungal
- Antiviral
- Alzheimer's disease inhibition
- Neuroprotective
- Anticonvulsant
- Phytoalexins
- Ulcerogenic
- antihypertensive





# The Specific Preparation of Honey Samples

Using the analytical balance, targetly 1 g (writting exact weights to the protocol) of the sample was weighted into a small beaker and dissolved in 10 ml of water to prepare dissolved honey (DH) samples by stirring with a glass rod. Results will be 10 times calculated. After complete dissolution of honey in water were samples prepared & extracted by using SPE columns then.



# External Calibrators Preparation

Using the analytical balance, roughly 40 mg coumarin + 40 mg coumaric acid (write they resulted weight) of the calibrator weigh into a convenient vial and dissolved in 5 ml of methanol (cca 4 mg/ml, CAL solution I). This stock will further mix 100  $\mu$ l of CAL solution I with 19,900 ml of pure methanol (CAL solution II, cca 4  $\mu$ g/ml; 4 000 ng/ml).

# ISTD Preparation

**Coumarin-d4 (5 mg)** and **coumaric acid (1 mg)** were dissolved in their own glass vials with 1 mL of 70% methanol (overnight in freezer). After complete reconstitution were **1 ml of coumarin (5 mg/ml)** vial and **1 ml of coumaric acid (1 mg/ml)** vial diluted with 98 ml of 70% methanol to prepare 100x diluted ISTD MIX I solution (**coumarin-d4 0,05 mg/ml** and **coumaric acid-<sup>13</sup>C<sub>3</sub> 0,01 mg/ml**, resp. **0,025 mg/ml** and **0,005 mg/ml**). Then, 10 ml of ISTD MIX I solution was diluted with 90 ml 70% methanol to prepare **ISTD MIX II solution (working solution)** with final concentrations: **coumarin-d4 0,05 µg/ml** and **2,5 µg/ml for coumaric acid-<sup>13</sup>C<sub>3</sub>**. Aliquotes of ISTD MIX I and ISTD MIX II are in freezer (à 500 µl). Concentrations of ISTD in prepared samples/calibrators were 5 ng/ml and 25 ng/ml.

# The Preparation of Coumarin STAD Samples

**STAD 0:** 0  $\mu\text{l}$  of CAL solution II + 100  $\mu\text{l}$  of ISTD MIX II + 900  $\mu\text{l}$  of unknown DH sample

**STAD 1:** 20  $\mu\text{l}$  of CAL solution II + 100  $\mu\text{l}$  of ISTD MIX II + 880  $\mu\text{l}$  of DH pool (unknown concentration of DH pool + 80 ng/ml of coumarin; coumaric acid)

**STAD 2:** 50  $\mu\text{l}$  of CAL solution II + 100  $\mu\text{l}$  of ISTD MIX II + 850  $\mu\text{l}$  of DH pool (unknown concentration of DH pool + 200 ng/ml of coumarin; coumaric acid)

**STAD 3:** 100  $\mu\text{l}$  of CAL solution II + 100  $\mu\text{l}$  of ISTD MIX II + 800  $\mu\text{l}$  of DH pool (unknown concentration of DH pool + 400 ng/ml of coumarin; coumaric acid)

**STAD 4:** 200  $\mu\text{l}$  of CAL solution II + 100  $\mu\text{l}$  of ISTD MIX II + 700  $\mu\text{l}$  of DH pool (unknown concentration of DH pool + 800 ng/ml of coumarin; coumaric acid)

# Phenomenex Strata-X 33 um Polymeric Reversed Phase 30mg / 1mL (8B-S100-TAK)

## Rules:

- ✓ be careful with optimal speed of elution (1 drop/1 second; 1drp/1sec)
- ✓ use 1 column in SPE manifold only
- give SPE column on one position in SPE manifold
- give wasting tube into SPE manifold
- wash column with **3 ml of methanol (1drp/1sec)**
- wash column with **3 ml of water (1drp/1sec)**
- take and apply **1 ml of prepared samples/calibrator** to column with elution speed **1drp/1sec**
- after sample application, remove wasting tube and give sample tube (convenient for further evaporation - 5 mL Eppendorf tubes) into SPE manifold
- wash the column with **3 mL of ethylacetate (1drp/1sec)**
- after elution, remove sample tube and give empty wasting tube into manifold (marked Erlenmeyer beaker for organic waste is in fume hood)
- all eluents were evaporated to dryness (RVC)

# p - coumaric acid internal calibrators

## (E)-p-coumaric acid

Molecular Formula  $C_9H_8O_3$

Average mass 164.158 Da

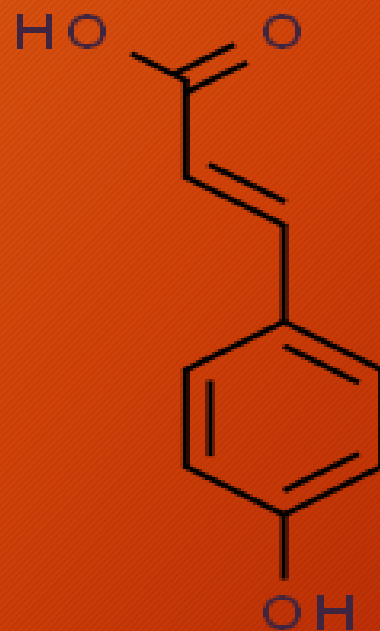
Monoisotopic mass 164.047348 Da

## p-coumaric acid-1,2,3- $^{13}C_3$

Molecular Formula  $^{13}C_3C_6H_8O_3$

Average mass 167.110 Da

Monoisotopic mass 167.05741 100



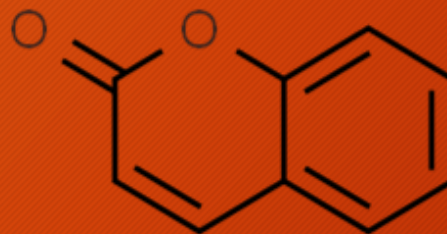
# coumarin internal calibrators

## Coumarin

Molecular Formula  $C_9H_6O_2$

Average mass 146.143 Da

Monoisotopic mass 146.036774 Da



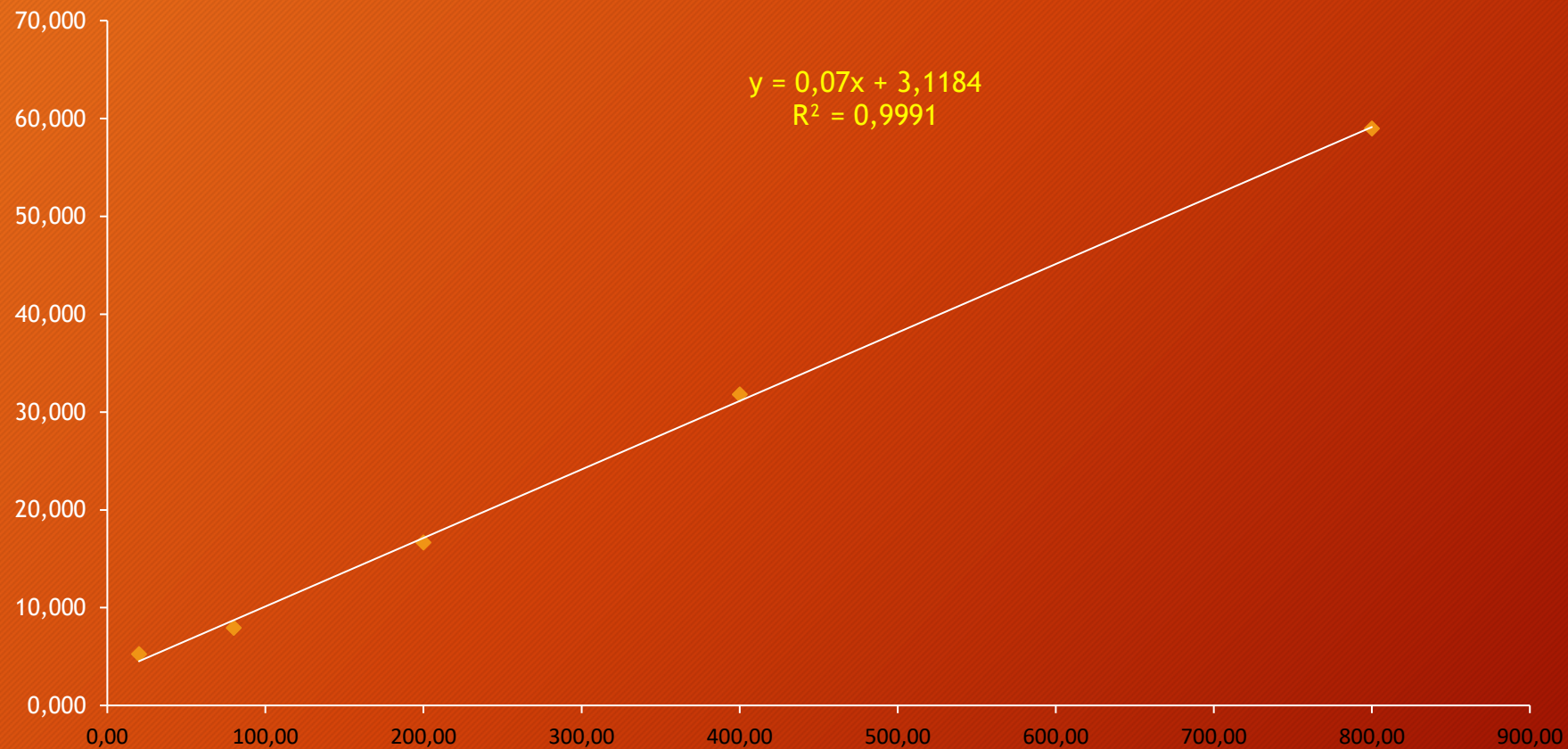
## Coumarin-d4

Molecular Formula  $C_9H_2D_4O_2$

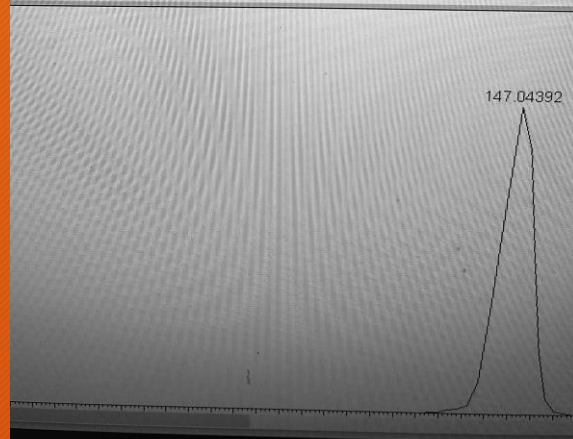
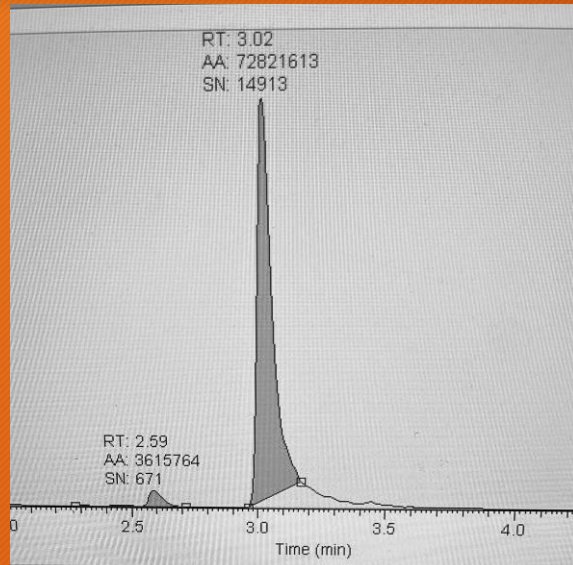
Average mass 151.070 Da

Monoisotopic mass 151.06933 Da

# Coumarin STAD calibration



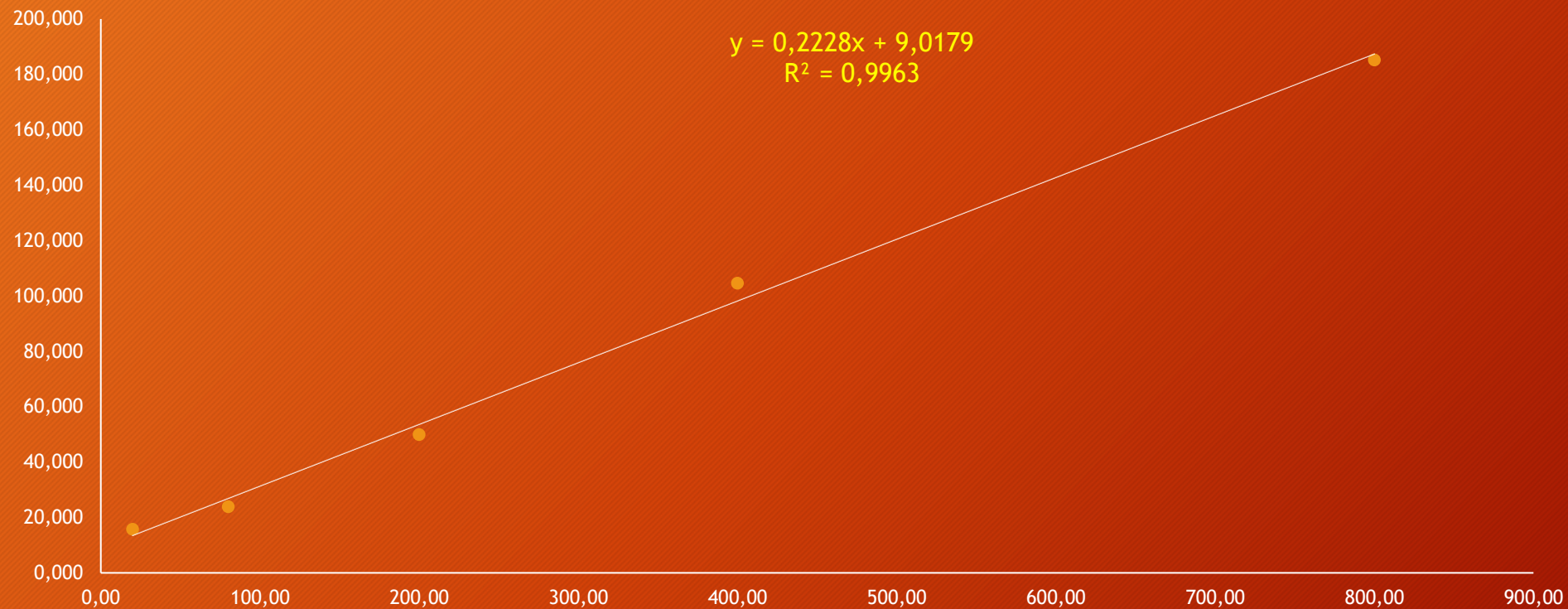
# coumarin results



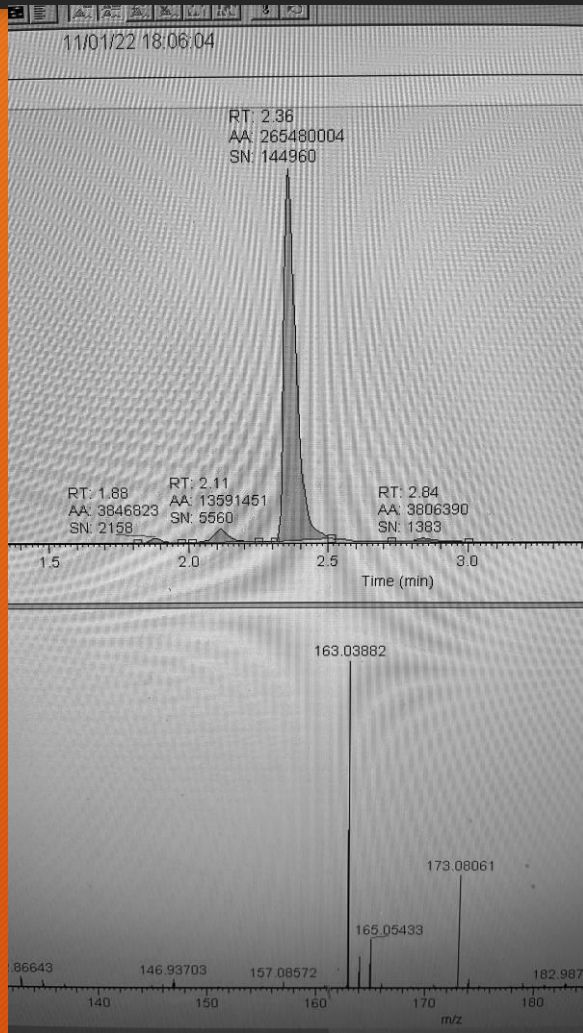
ng/ml	µg/g	Sample No
33,99	342,00	A3
6,60	66,26	A1
8,67	86,96	A2
11,92	119,57	A4
12,66	127,18	A5
12,40	80,23	N1
24,66	247,19	N2
4,19	42,33	P1
5,90	59,51	P2
17,99	181,34	P3
8,11	81,29	P4
1,05	10,48	P5
2,80	28,13	P6
4,04	40,54	P7
1,57	15,73	P8
5,08	51,30	Z4
6,63	66,80	Z5
3,29	33,44	Z1
3,51	35,33	Z2
6,67	67,39	Z3



# P-coumaric acid STAD Calibration



# p-coumaric results



ng/ml	µg/g	Sample No.
5,45	54,66	1
28,92	289,32	2
6,27	62,87	3
6,15	61,95	4
324,34	3263,12	5
214,34	2157,19	6
9,66	96,64	7
128,27	1293,09	8
14,86	149,45	9
9,34	93,97	10
7,01	70,18	11
17,40	175,64	21a
25,64	257,67	21b
22,22	222,49	21c

# Conclusions and Prospectives

- Monitoring of coumarin levels can be used to determine the risk of honey toxicity
- Honey is not a food, but a food supplement with certain medicinal/toxical potential
- The ratio of coumaric acid to the original coumarin corresponds to the degree of additional treatment of the honey by the beekeeper
- Coumaric acid levels as a measure of honey bioquality
- Tracking other coumarins as a challenge for the future