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Keracol
Functional, natural, sustainable

Scalable anthocyanin extraction and purification methods for industrial applications

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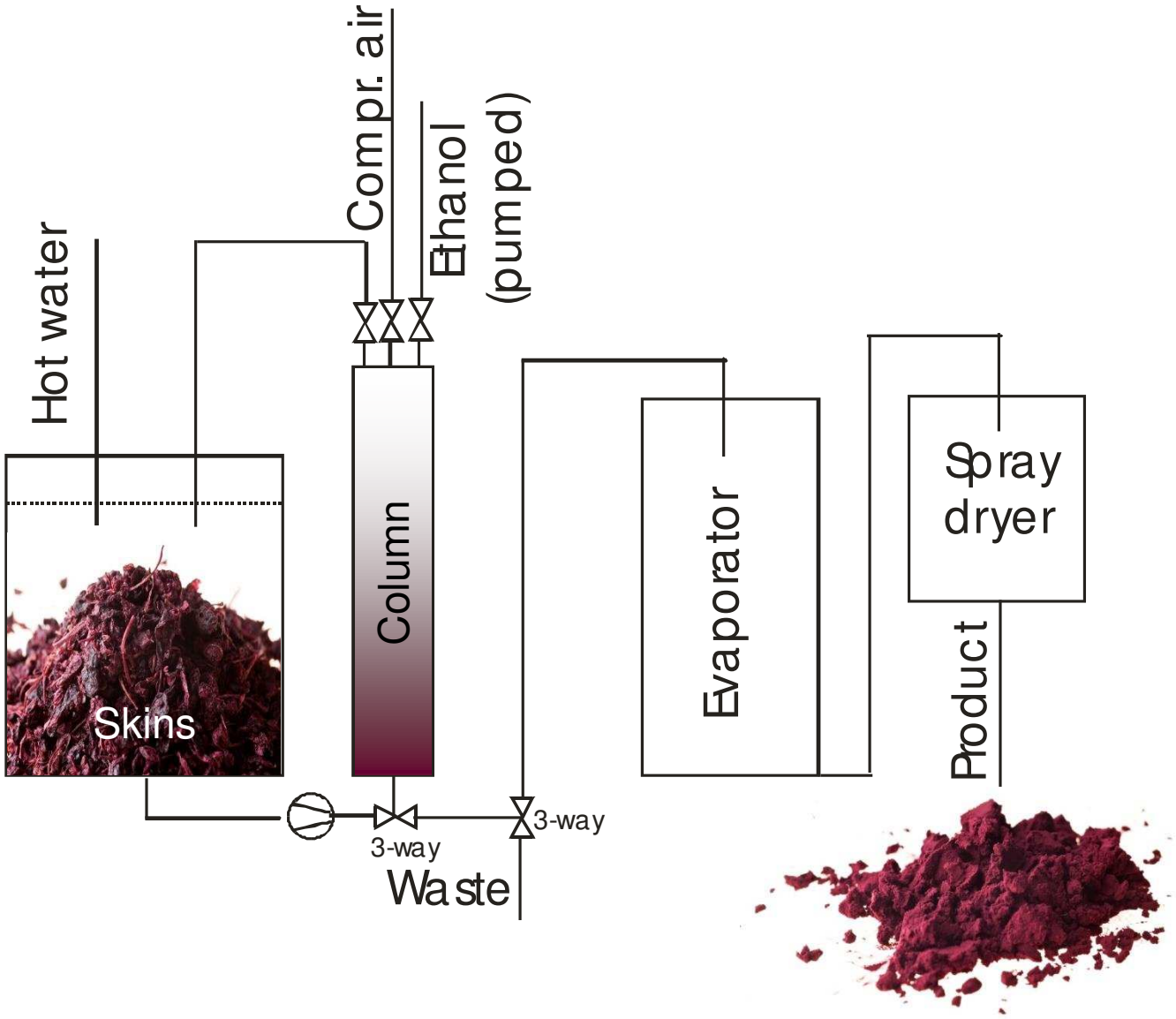
Extraction-Purification

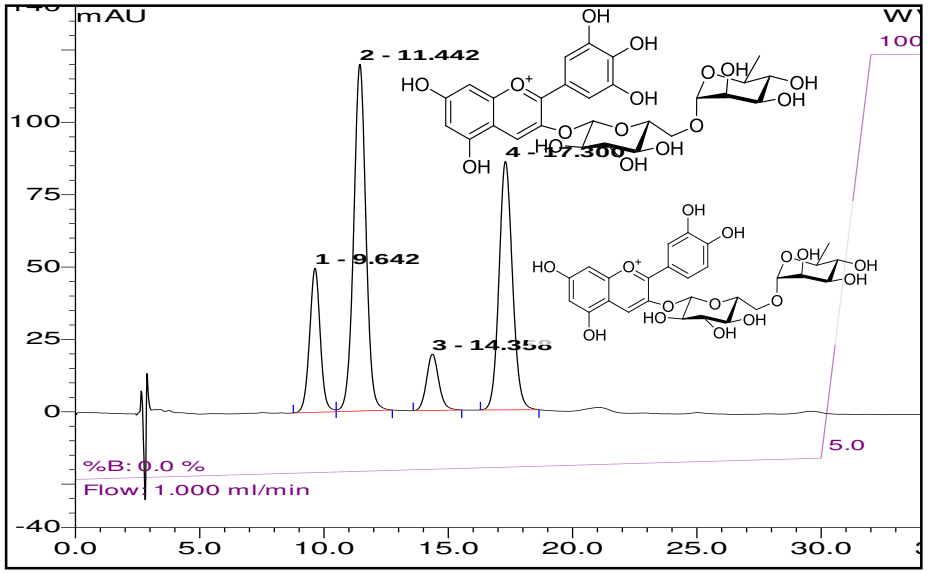


Industrial-scale process

- Extract from berry waste
- Fruits are used in juice production
- Only waste skins are used to produce the extract
- Sustainably sourced
- Acidic aqueous extraction of dried skins
- Concentration using Solid Phase Extraction (SPE)
  - resin removes free sugars and small organic acids
- Ethanol elution of polyphenols retained by resin

waste

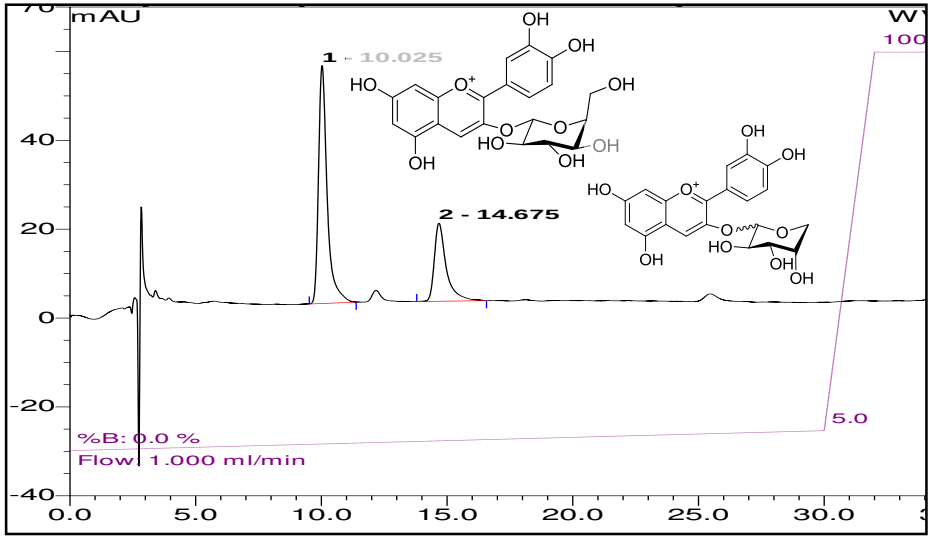
BLACKCURRANT (*Ribes nigrum*)



Cy3glc (7.0%)
Cy3rut (34.0%)
Dp3glc (15.7%)
Dp3rut (43.3%)

waste

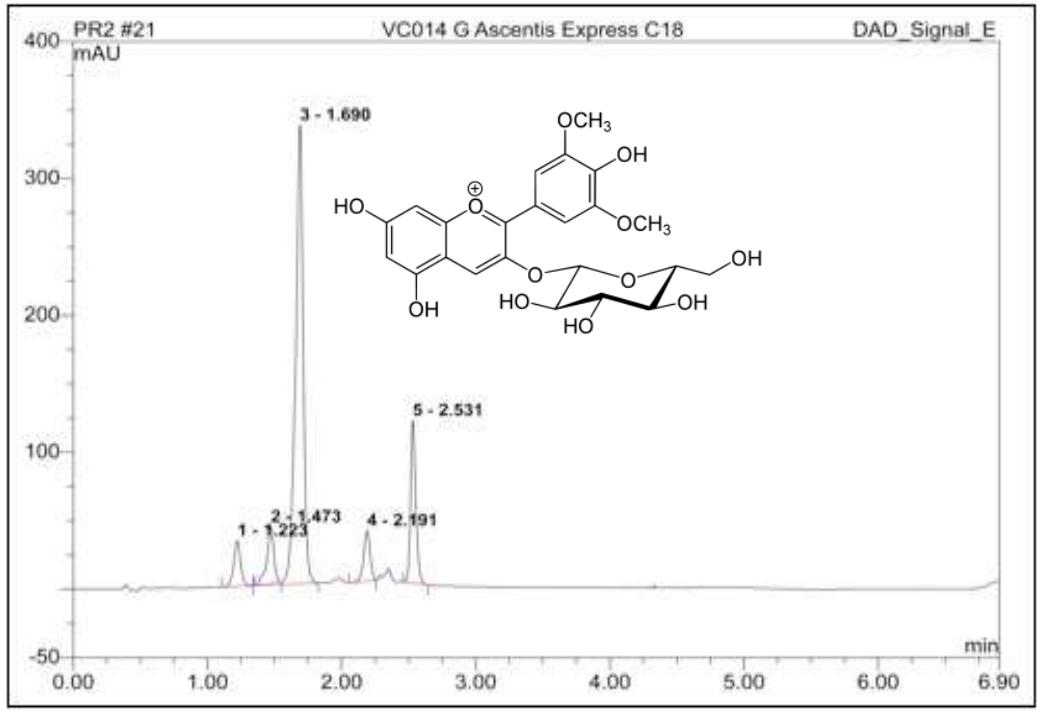
ARONIA (*Aronia melanocarpa*)



Cy3gal (68%)
Cy3arab (30%)

waste

GRAPE (*Vitis vinifera*)



Cy3glc (5.8%)
Dp3glc (7.3%)
Mv3glc (65.3%)
Pn3glc (6.0%)
Pt3glc (15.6%)

Case Study 1: Natural hair dyes

- ANC extract from blackcurrant waste skins (UK grown)
- Patented (WO2010131049) semi-permanent hair colorants and coloration process
- Range of shades, fast to 12+ washes

Case Study 2: Food colorants

- ANC extracted from sustainable sources
- Lake pigment formed with metal
- Pigments in range of colours
- Both water soluble and water insoluble pigments possible

Case Study 3: Marking eggshell

- Inks using ANC extracted from waste (WO2015128646)
- Increase in the information placed on an egg
- Reduced environmental and toxicological impact
- Enhance security, safety, and traceability

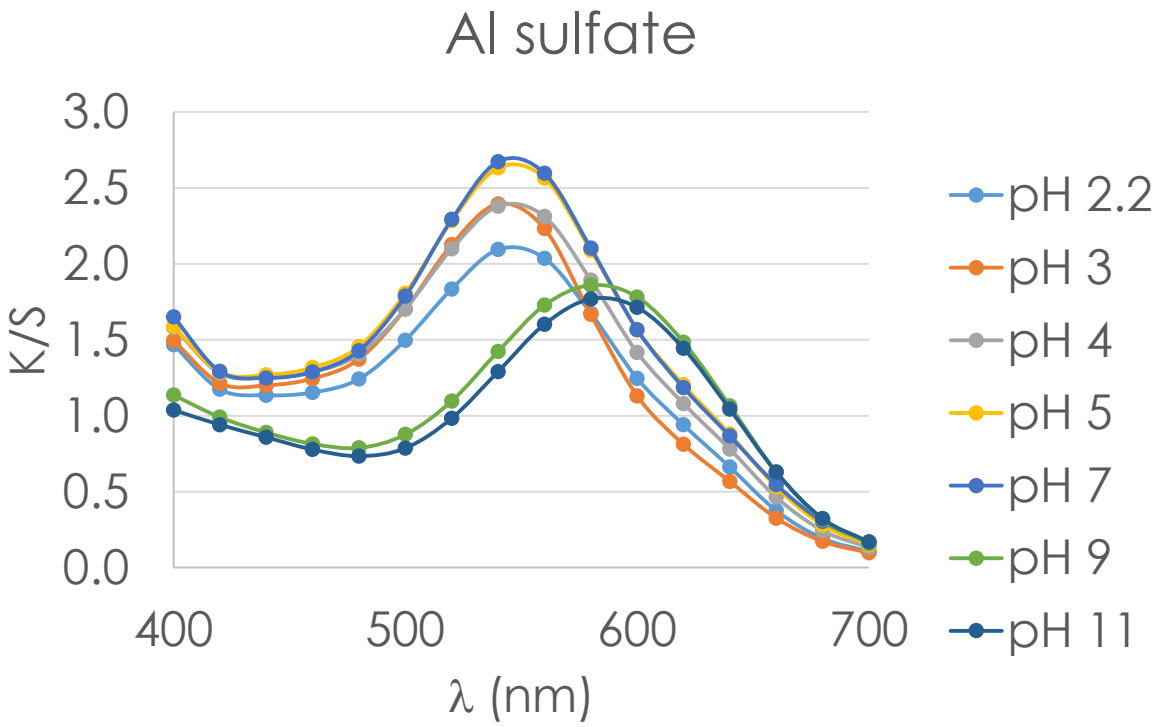
Current research on ANC applications

- Cosmetic colorants and make-up
- Food colorants for lipophilic media
- Coloration of high-value textiles
- Collaboration with University of Porto

U.PORTO

Dyeing silk and wool with blackcurrant anthocyanins

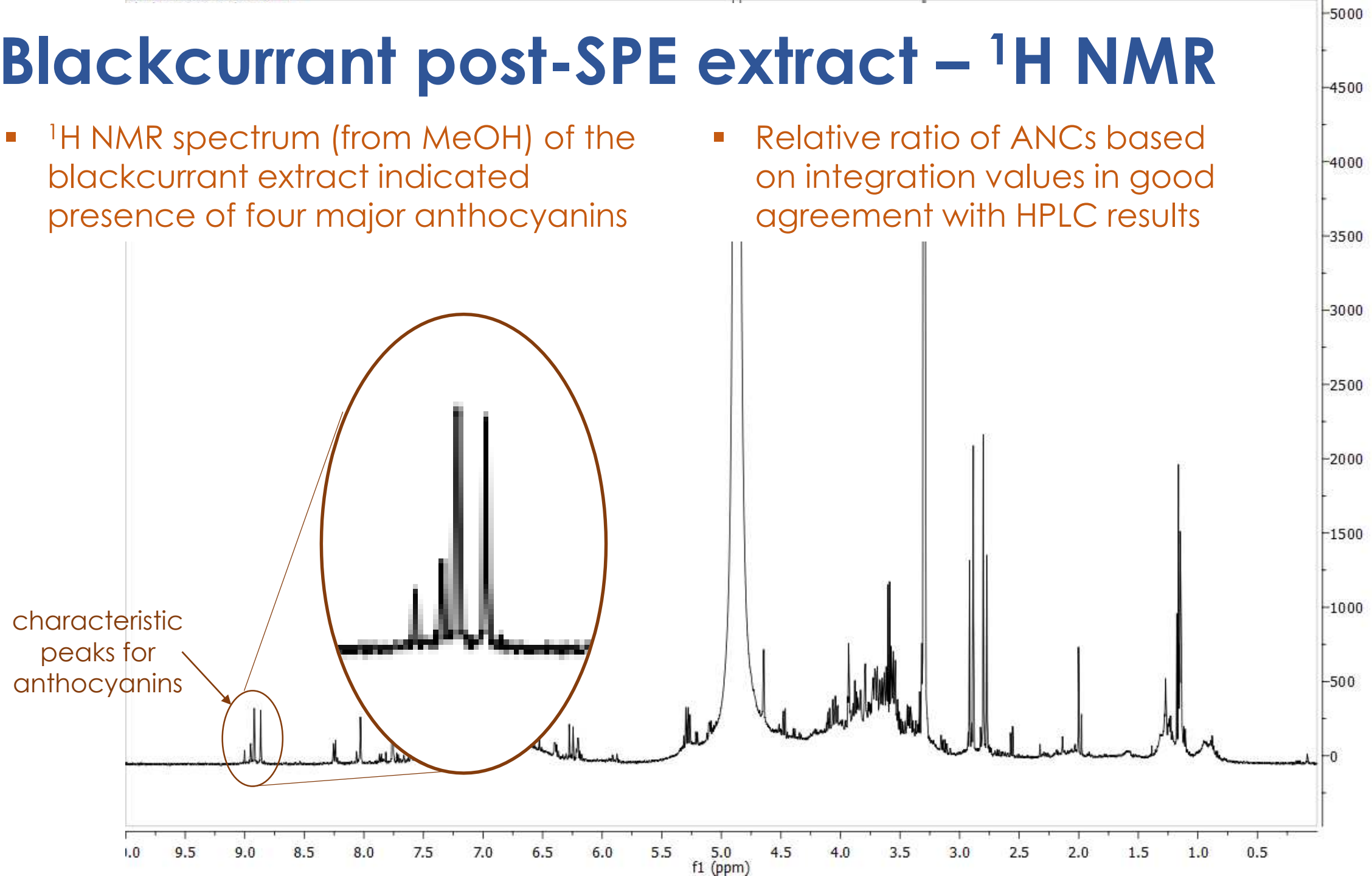
- Range of colours obtainable by modifying pH and mordant metal
- Good wash fastness (30 °C)
- Poor to medium light fastness



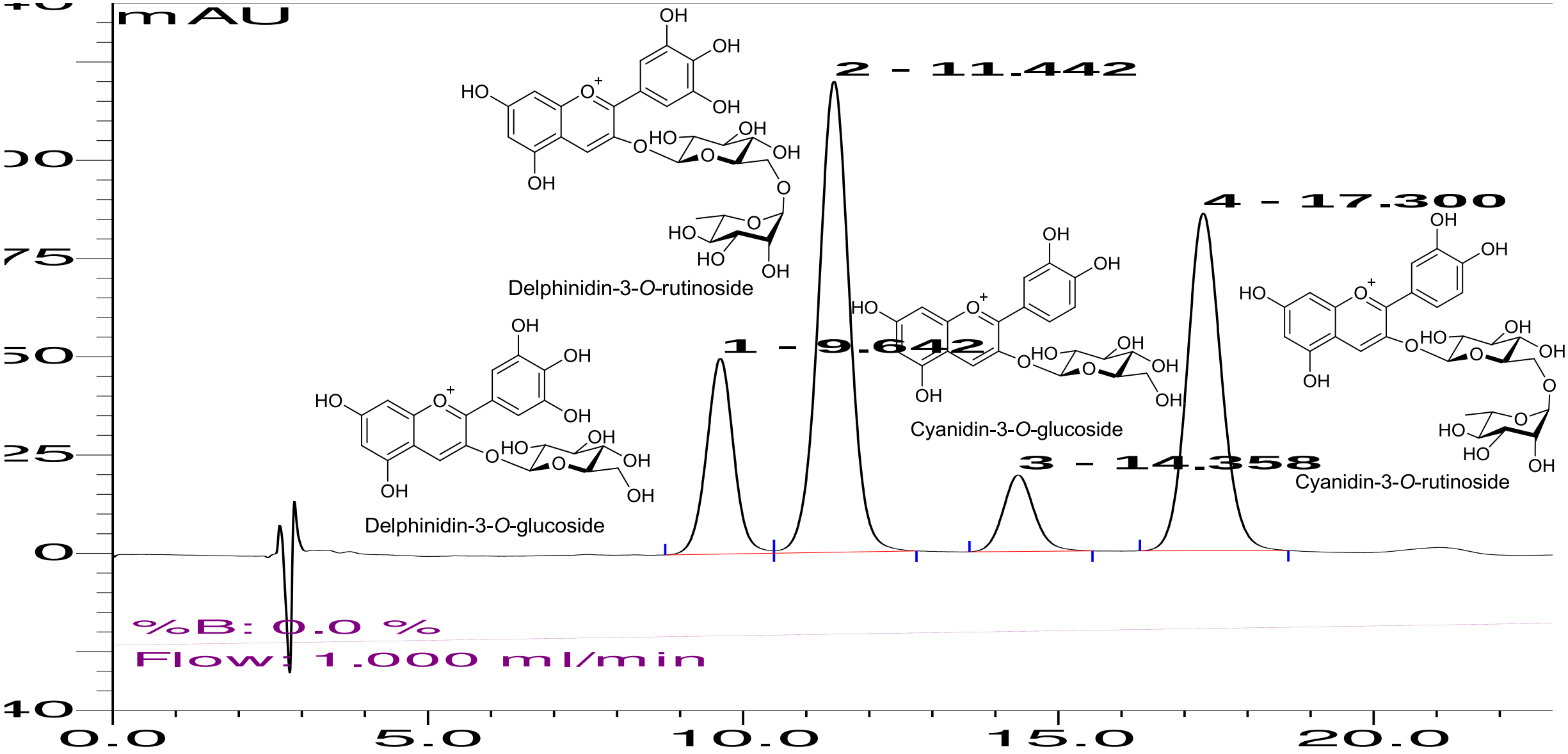
Investigation of the full composition of blackcurrant skin extract post-SPE

Blackcurrant post-SPE extract – ^{1}H NMR

- ^{1}H NMR spectrum (from MeOH) of the blackcurrant extract indicated presence of four major anthocyanins
- Relative ratio of ANCs based on integration values in good agreement with HPLC results

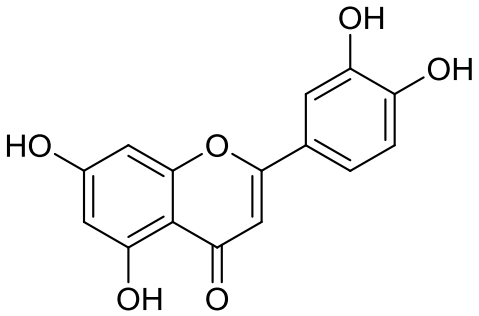


Anthocyanin-rich extract

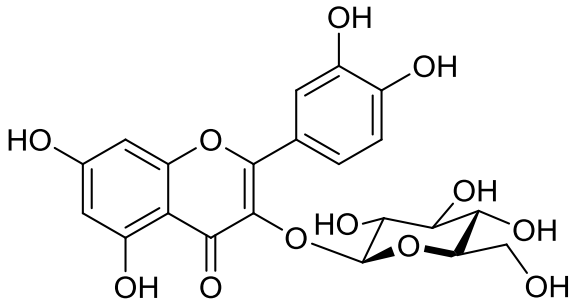


Identification of other components

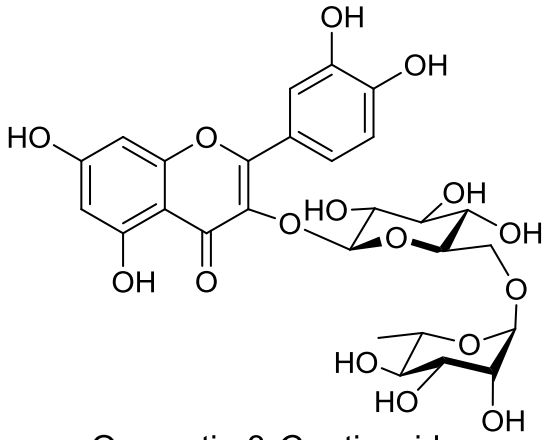
FLAVANOLS:



Quercetin

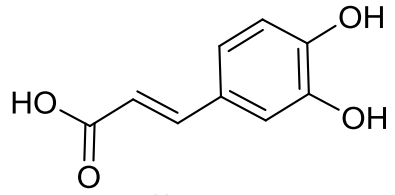


Quercetin-3-*O*-glucoside

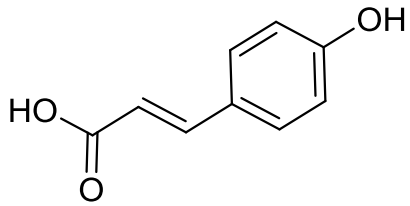


Quercetin-3-*O*-rutinoside

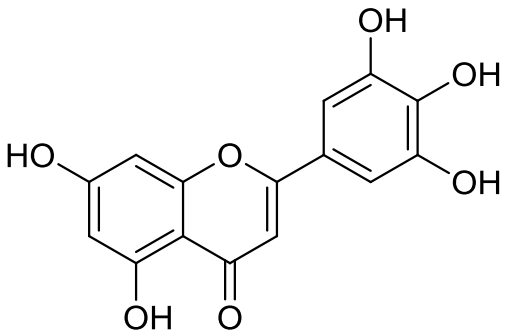
HYDROXYCINNAMIC ACIDS:



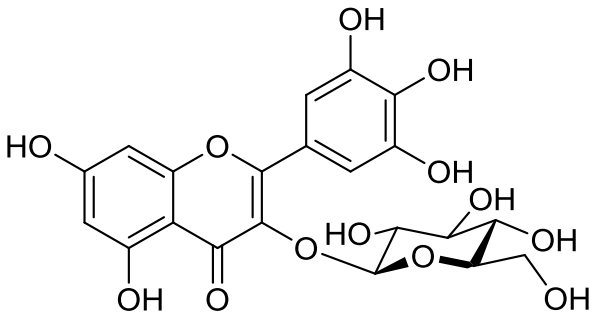
caffeic acid



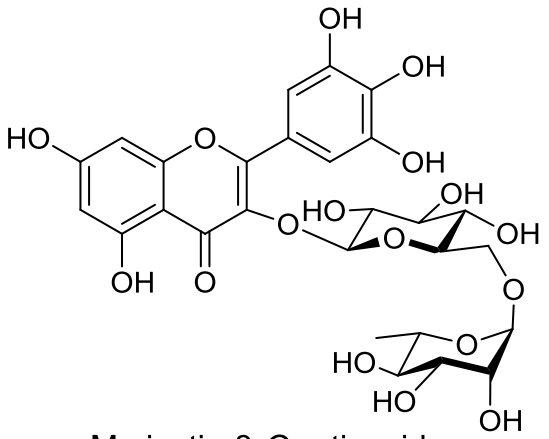
p-coumaric acid



Myricetin



Myricetin-3-*O*-glucoside



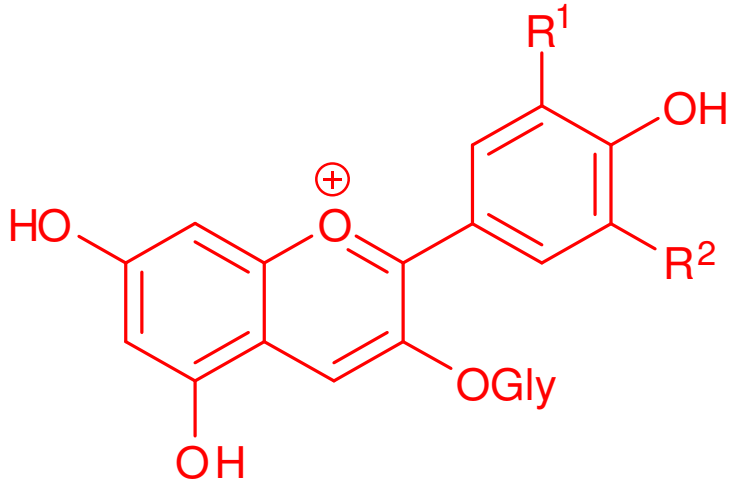
Myricetin-3-*O*-rutinoside

Liquid-liquid partitioning

- Aim was to purify anthocyanin extract by separating from other polyphenols
- Method for anthocyanin enrichment had to be scalable and economically viable
- Liquid-liquid partitioning was considered a good starting point as it can be scaled up relatively easily

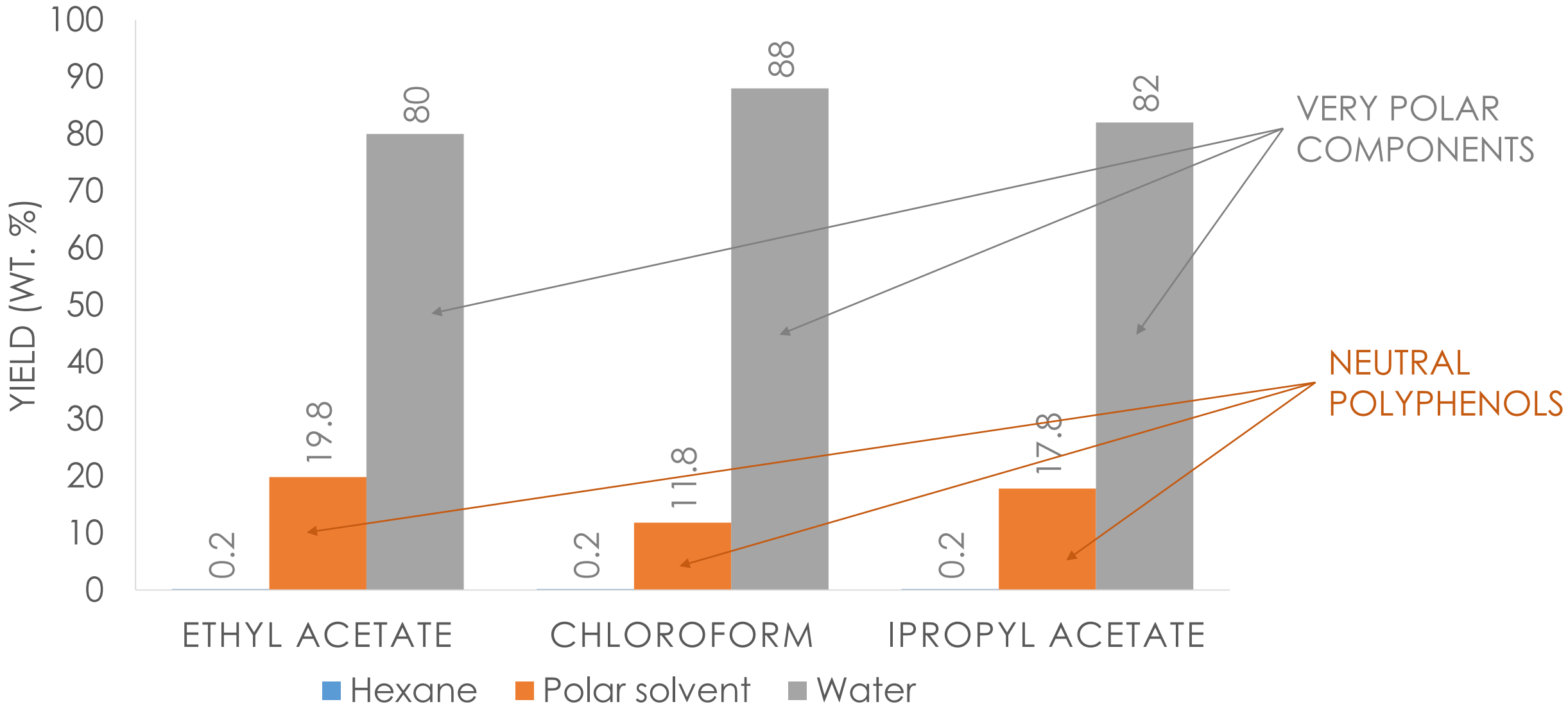
Liquid-liquid partitioning

- In acidic conditions, charged AH^+ anthocyanins have higher solubility in water
- However, uncharged flavonoids are expected to be relatively less water soluble
- Sugar moieties should enhance water solubility of neutral polyphenols

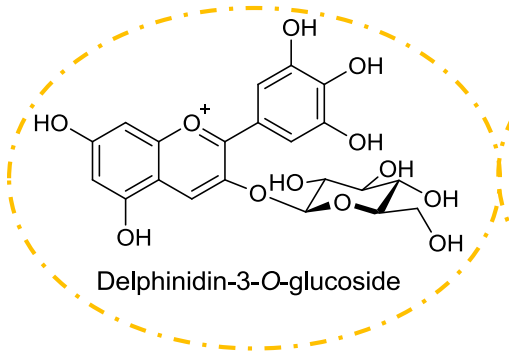


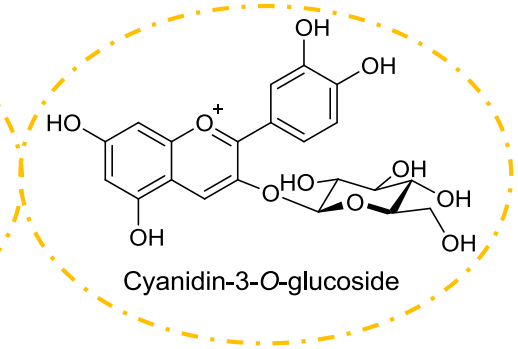
flavylium cation (AH^+)

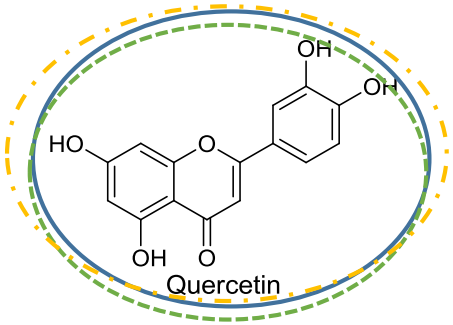
Selected solvents: hexane, ethyl acetate, *i*propyl acetate and chloroform, partitioning with acidified water (pH 1.8; HCl)

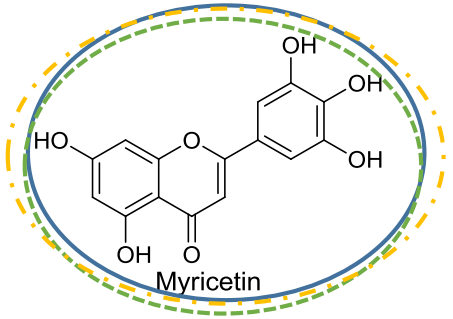


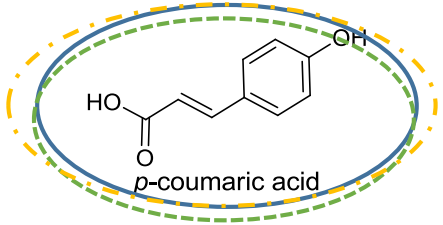
Liquid-liquid partitioning

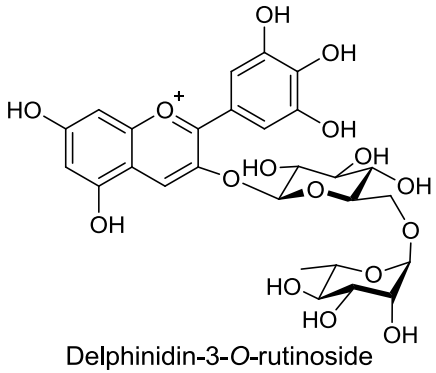


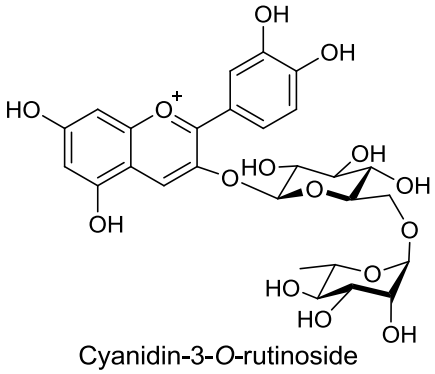


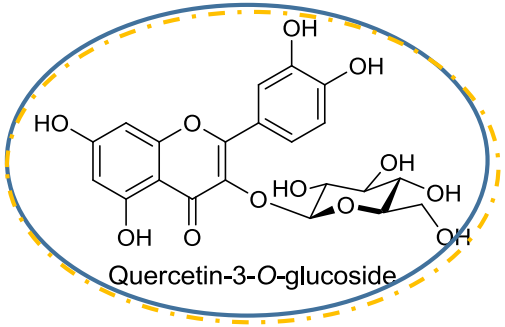


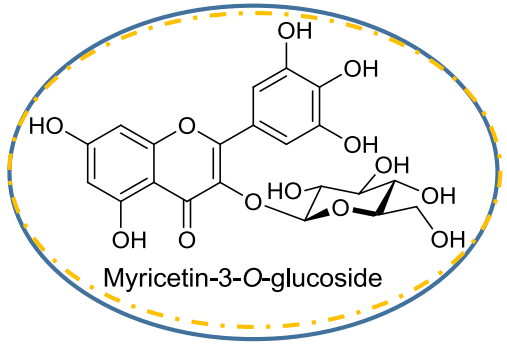


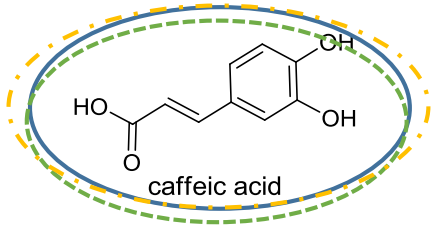


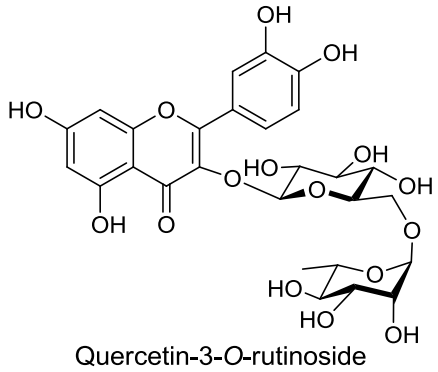


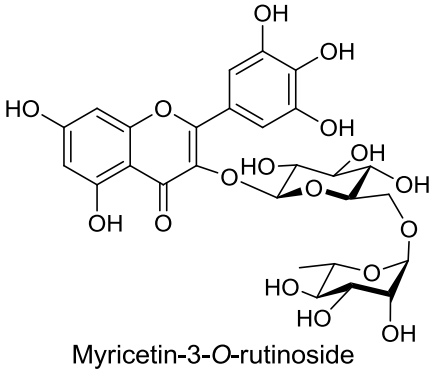












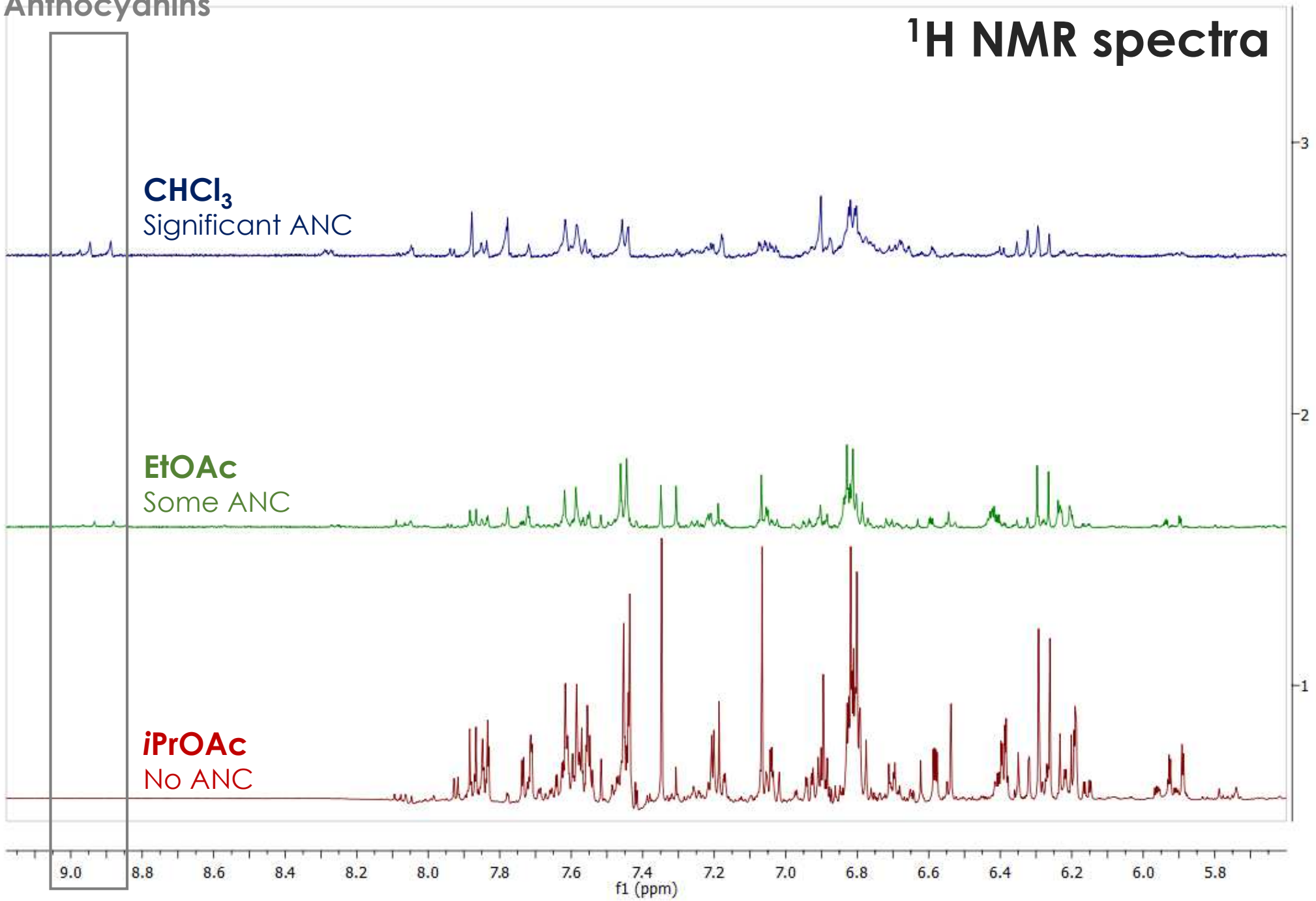
ethyl acetate

*i*propyl acetate

chloroform

Anthocyanins

^{1}H NMR spectra

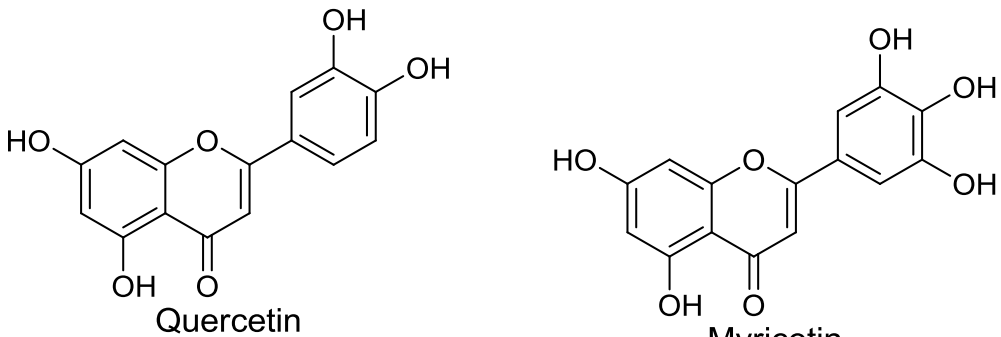


Sequential liquid-liquid partitioning

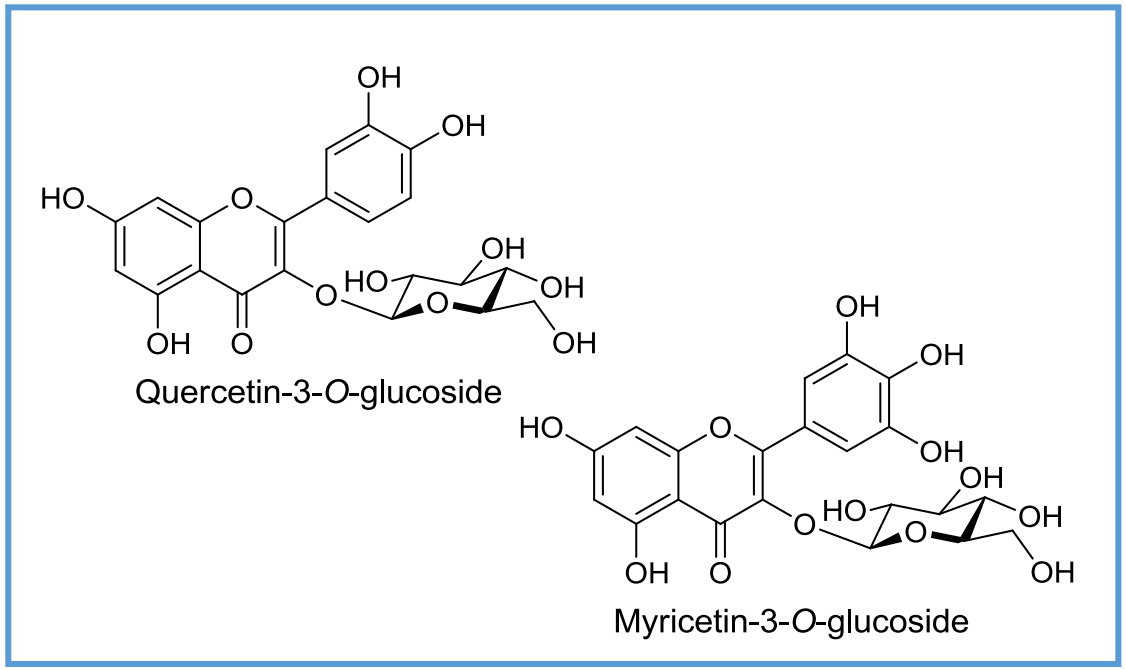
- Extract first partitioned with isopropyl acetate
  - removes quercetin and myricetin algycons and hydroxycinnamic acids

- Extract then partitioned with ethyl acetate
  - removes quercetin and myricetin glucosides

*i*propyl acetate

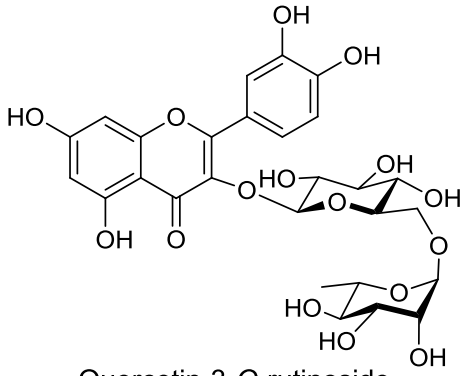


ethyl acetate

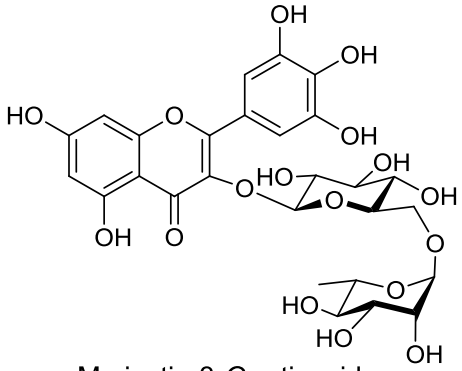


pH-controlled liquid partitioning

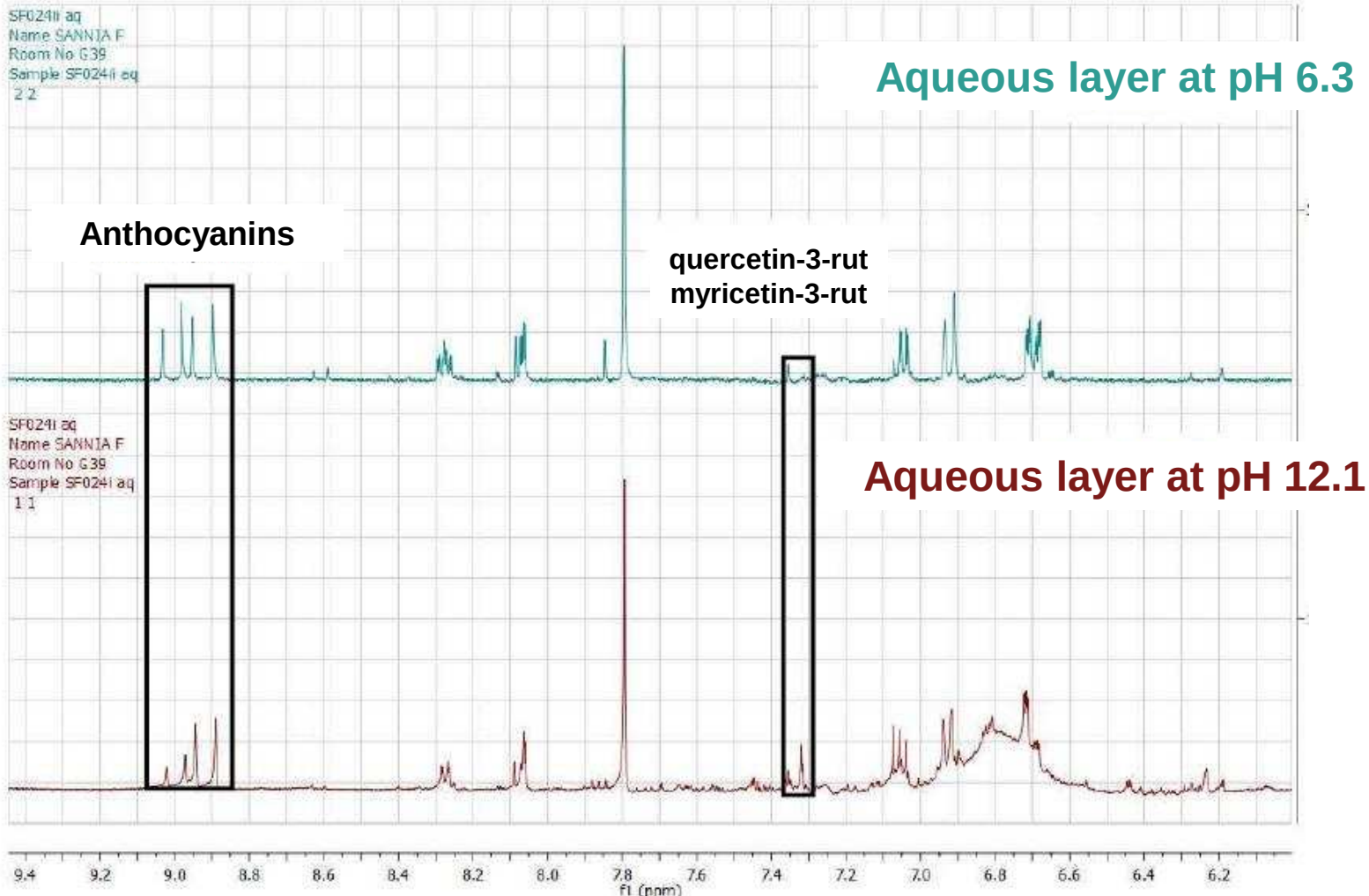
- At pH 6.3: quercetin rutinoside (7.52 ppm) and myricetin rutinoside (7.30 ppm) migrate into EtOAc layer
- At pH 12.1: quercetin and myricetin rutinosides do not migrate into EtOAc

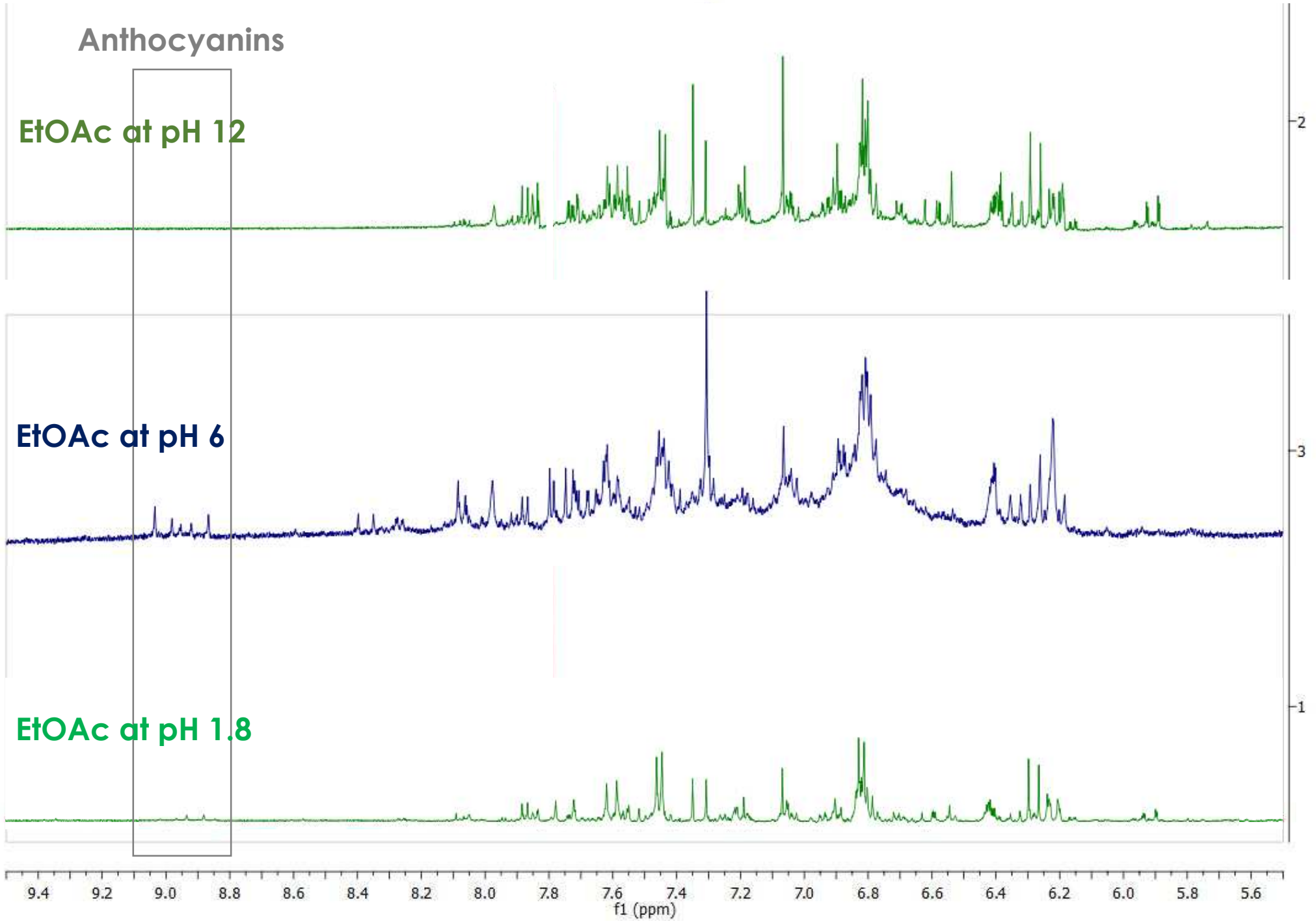


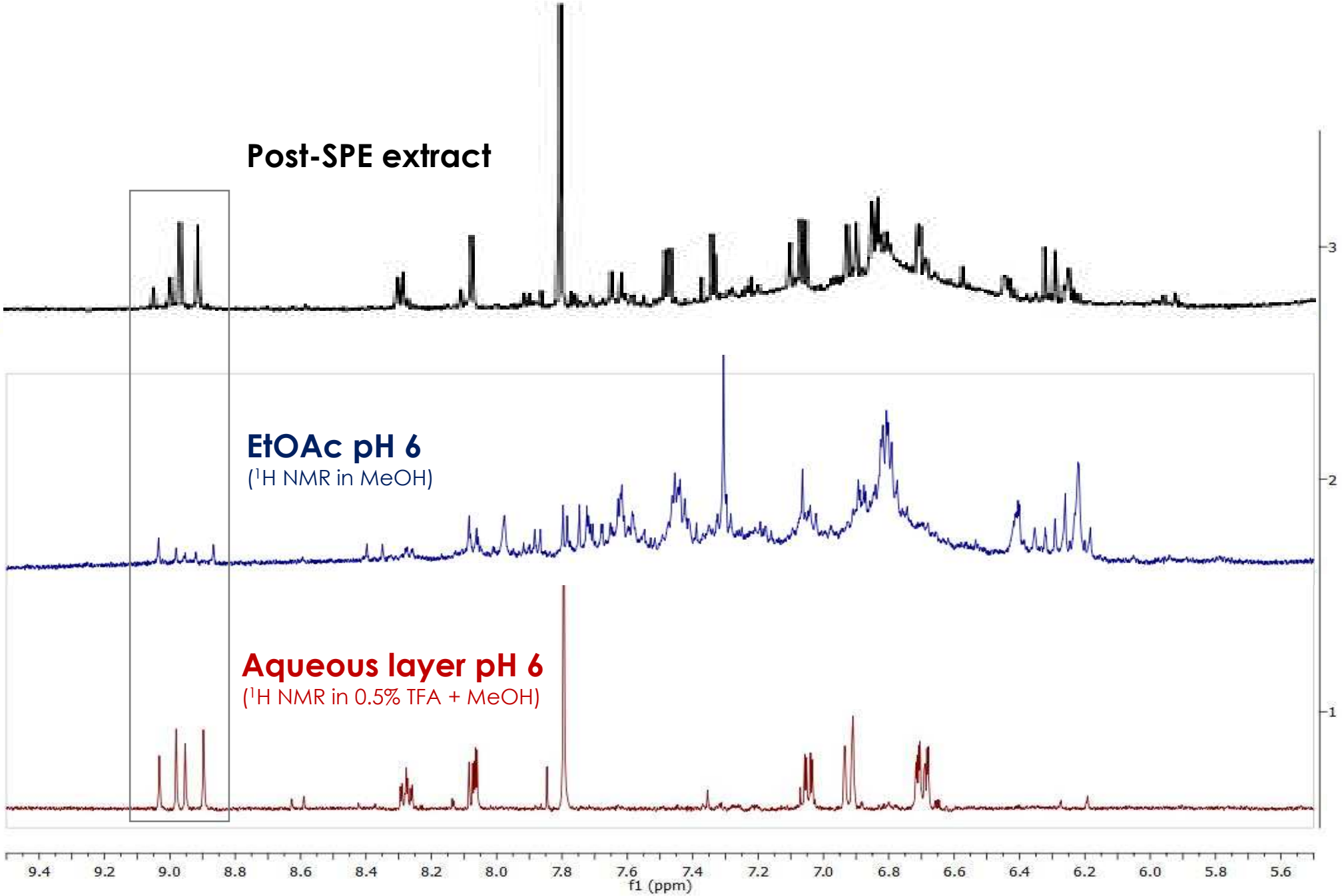
Quercetin-3-*O*-rutinoside



Myricetin-3-*O*-rutinoside




Anthocyanins
EtOAc at pH 12
EtOAc at pH 6
EtOAc at pH 1.8
2
3
1
9.4
9.2
9.0
8.8
8.6
8.4
8.2
8.0
7.8
7.6
7.4
7.2
7.0
6.8
6.6
6.4
6.2
6.0
5.8
5.6
f1 (ppm)


Post-SPE extract
EtOAc pH 6
(1H NMR in MeOH)
Aqueous layer pH 6
(1H NMR in 0.5% TFA + MeOH)
3
2
1
9.4
9.2
9.0
8.8
8.6
8.4
8.2
8.0
7.8
7.6
7.4
7.2
7.0
6.8
6.6
6.4
6.2
6.0
5.8
5.6
f1 (ppm)

Sequential liquid-liquid partitioning with pH changes

BC extract comprises 48% ANC and 26% other polyphenols

caffeic acid

Delphinidin-3-*O*-glucoside

p-coumaric acid

Myricetin

Quercetin

Cyanidin-3-*O*-glucoside

Quercetin-3-*O*-rutinoside

Quercetin-3-*O*-glucoside

Myricetin-3-*O*-glucoside

Delphinidin-3-*O*-rutinoside

Myricetin-3-*O*-rutinoside

Cyanidin-3-*O*-rutinoside

Four ANC remain in aqueous layer (immediately acidified and freeze dried)

Myricetin and quercetin rutinosides removed with **EtOAc at pH 6.3**

Trace ANC

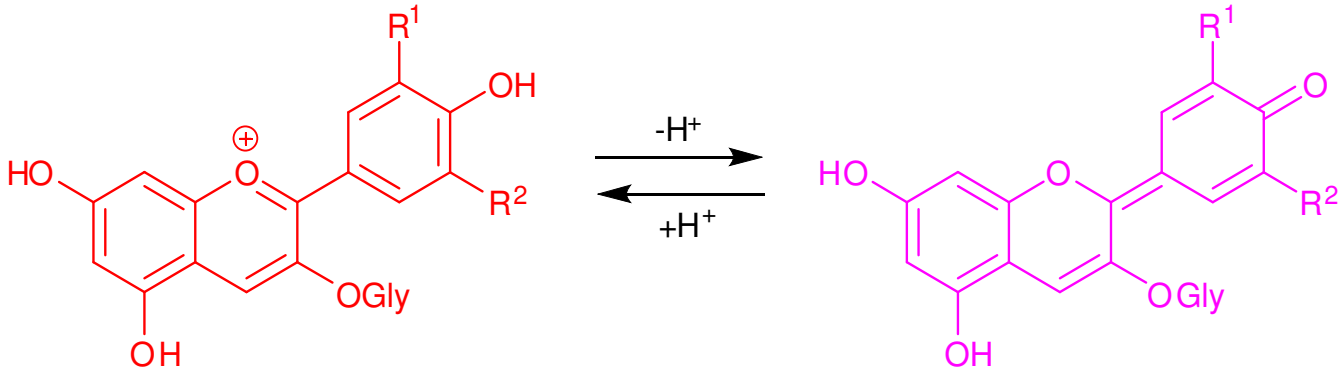
***i*PrOAc at pH 1.8** removes hydroxycinnamic acids and myricetin + quercetin aglycons

EtOAc at pH 1.8 selectively removes myricetin + quercetin glucosides

pH-controlled liquid partitioning

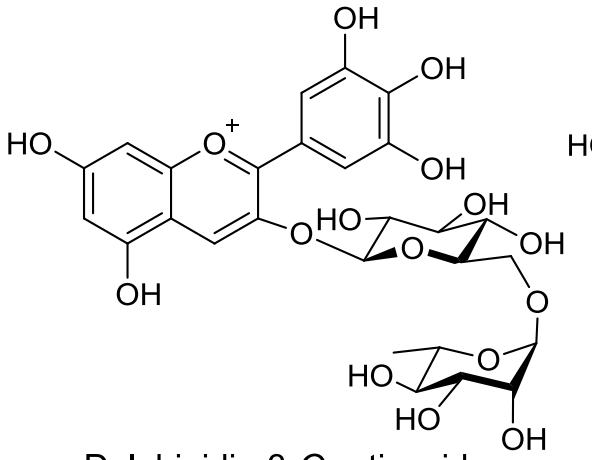
- At pH 6.3 myricetin-3-rut + quercetin-3-rut migrate into EtOAc

- At pH 6.3 neutral ANC anhydrobase (A) should form
  - But Cy-3-rut, Cy-3-glc, Dp-3-rut and Dp-3-glc (A form?) do not migrate into EtOAc
  - Potential of disruption of co-pigmentation? Breaking down interaction between ANC and myricetin/quercetin rutinosides?

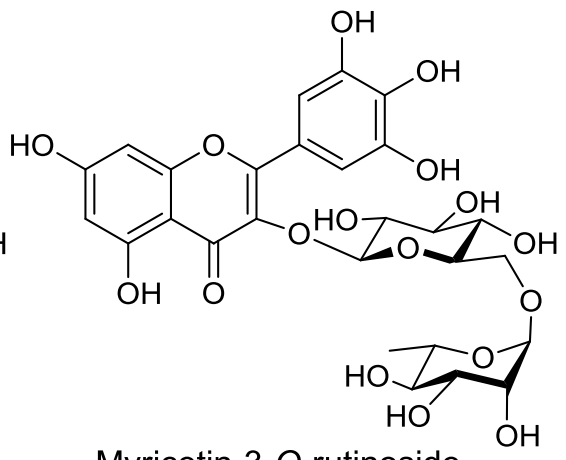


flavylium cation (AH^+)

anhydrobase (A)



Delphinidin-3-*O*-rutinoside



Myricetin-3-*O*-rutinoside

Conclusions

- Blackcurrant extract post-SPE can be purified using sequential liquid-liquid and pH change extraction
- Provides an isolate with only four anthocyanins present (clean ^{1}H NMR evidence) in a process that is industrially scalable
- Separation enable by relative water solubility of different components in BC extract mixture
- Further investigation required to determine reasons for EtOAc separation of myricetin-3-rut + quercetin-3-rut from neutral anhydrobase forms of ANC glycosides at pH 6.3

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