New Analytical Tools for the Study of Bioactive Polyphenols in *Prunus avium* L. (sweet cherry) and Evaluation of their Anti-oxidant Activity on an *In vivo* Model

Dr. Virginia Brighenti, Ph.D.

Department of Life Sciences, University of Modena and Reggio Emilia

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Summary

1. **Background and aim**

2. **Analytical characterisation**
    - **Conventional analytical techniques (HPLC-UV-ESI-MS and MS^2)**
    - **Electrochemical analysis (sensors)**

3. ***In vivo* assessment of the anti-oxidant activity on *C. elegans* model**

In collaboration with the research group of Prof. Celestino Santos-Buelga of the University of Salamanca

Prunus avium L. I.D.

- **Family:** ***Rosaceae***
- **Genus:** ***Prunus***
- **Species:** ***avium***
- **Nickname:** **sweet cherry**
- **Fruit:** **drupe**

Inside sweet cherry fruit

Polyphenols

Sugars

Organic acids

Vitamins

«Let food be your medicine and medicine be your food»
- Hippocrates

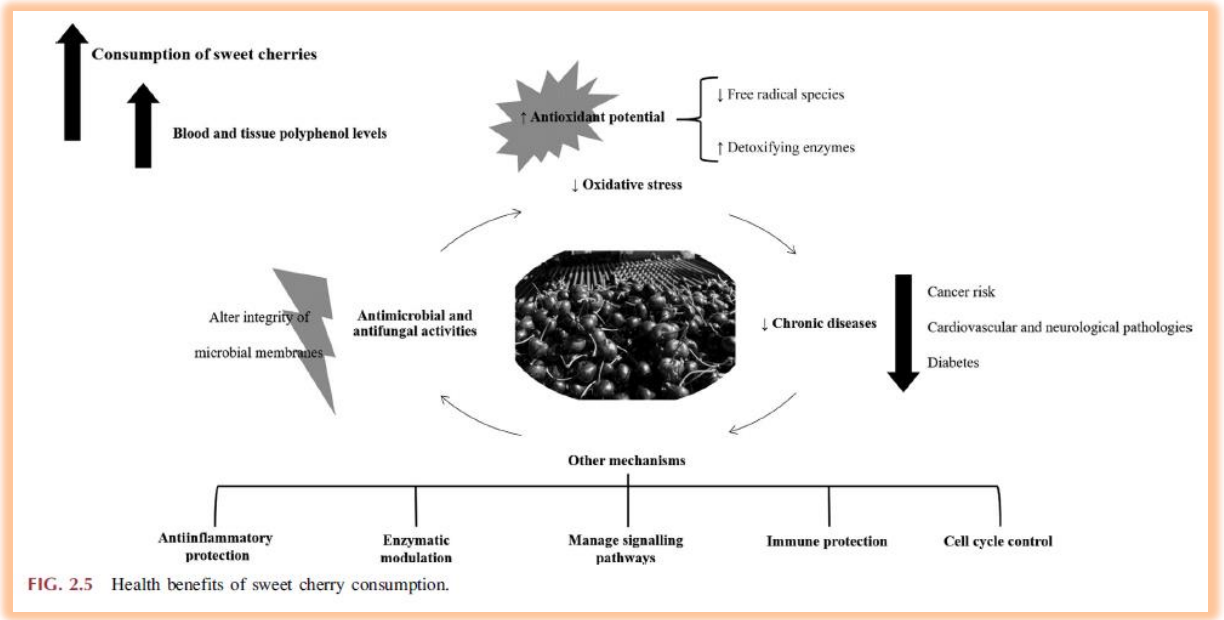

FIG. 2.5 Health benefits of sweet cherry consumption.

Gonçalves et al., Sweet Cherry Phenolic Compounds: Identification,Characterization and Health Benefits, in: Atta-ur-Rahman (Ed.), Studies in Natural Products Chemistry, Elsevier Inc., Amserdam, 2018.

Our goal

- Application of suitable analytical techniques to the **characterization** of sweet cherry polyphenolic fraction **and development of a fast and innovative tool** alternative to conventional techniques.

- Evaluation of the capacity of sweet cherry extract to increase the **resistance to thermal stress** in *C.elegans in vivo* model.

Sample

In this work, cherries belonging to the variety «**Moretta**» were considered. The sample was harvested near the city of Vignola in June 2018 and kept at -20 °C until the analysis.

Highlights

1. Background and aim

2. **Analytical characterisation**
   - **Conventional analytical techniques (HPLC-UV-ESI-MS and MS^2)**
   - **Electrochemical analysis (sensors)**

3. *In vivo* assessment of the anti-oxidant activity on *C. elegans* model

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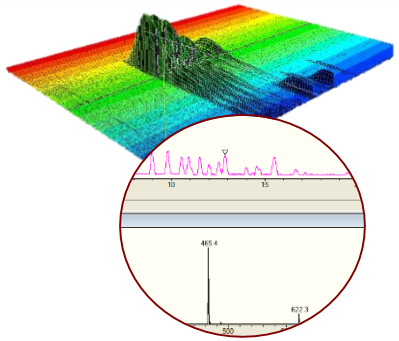
Workflow

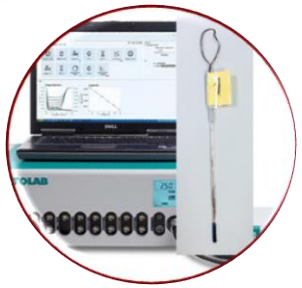
Extraction of secondary metabolites

Quantification of the main classes of polyphenols (Uv-Vis)

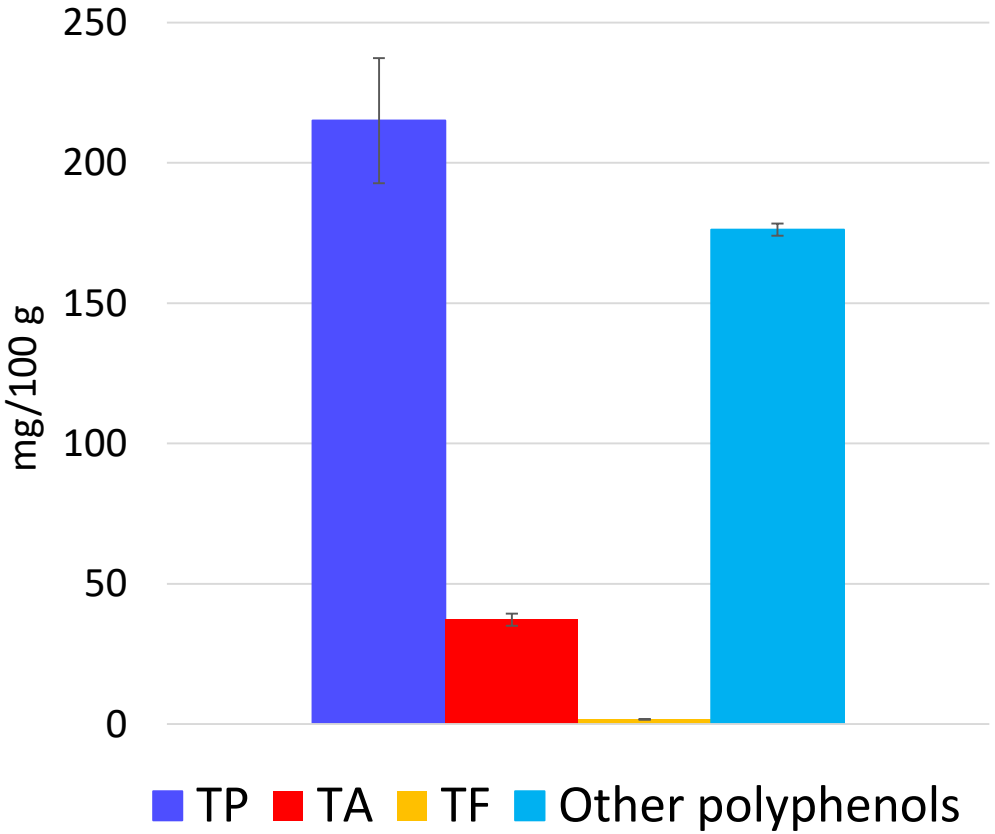
Identification of the compounds (HPLC-UV/DAD and MS and MS^2)

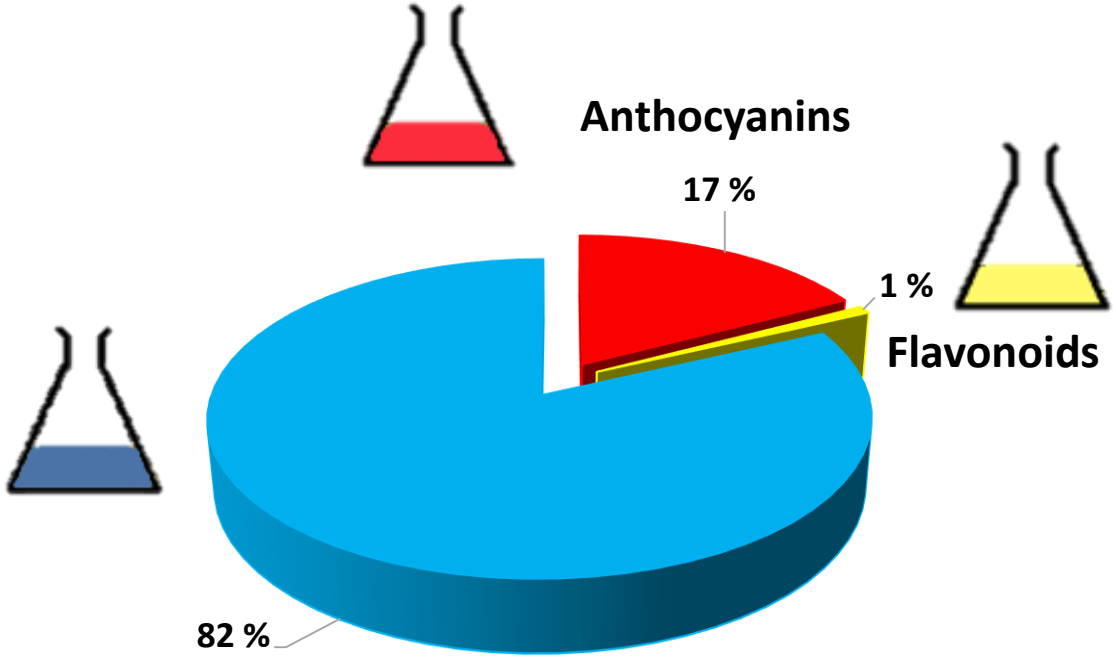
Electrochemical analysis





Spectrophotometric analyses





Which anthocyanins?
Which flavonoids?
Which other phenolics?

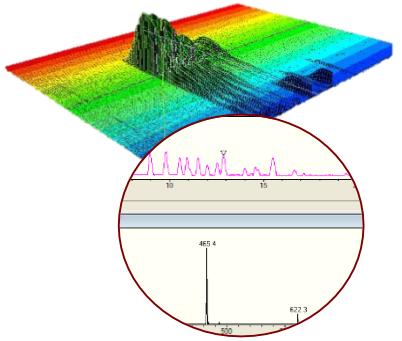
Compounds identification

HPLC-ESI-UV/DAD and MS and MS^2 analysis: combining spectral, MS and fragmentation information.

Chromatographic parameters

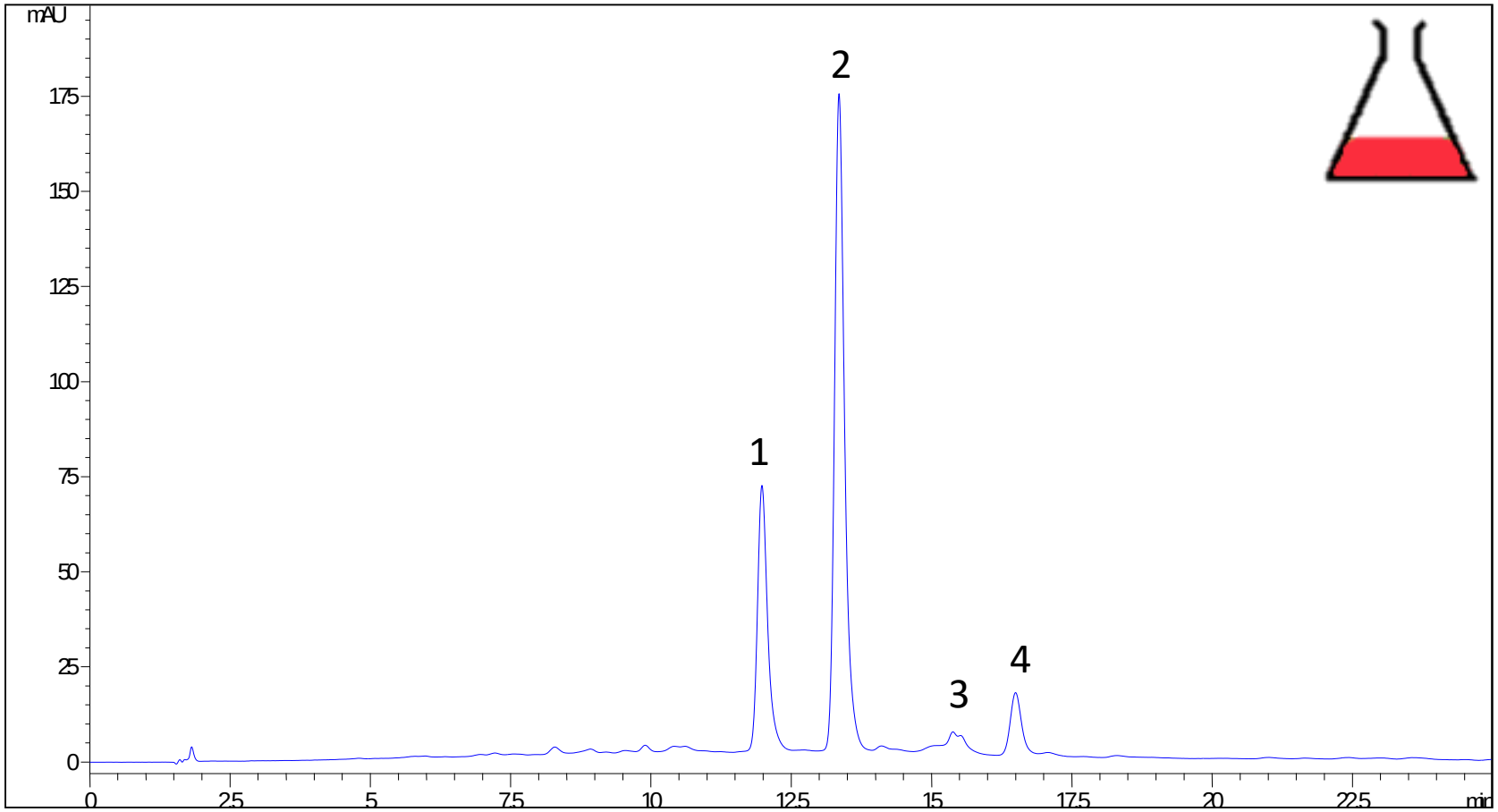
Chromatographic apparatus: HPLC Agilent Technologies modular model 1100;
Column tested: Zorbax C_{18} SB (150 x 4.6 mm, 5 µm, Agilent Technologies),
Mobile phase:
A) H_2O-HCOOH (9:1, v/v),
B) MeOH-H_2O-HCOOH (5:4:1, v/v/v), gradient elution;
Injection volume: 5 µL;
Flow: 1.0 mL/min (for MS and MS^2 analysis flow was splitted 1:5);
Column temperature: 25˚C;

Detection parameters



UV/DAD detection: 280 nm and 320 nm (phenolic acids and flavonoids), 520 nm (anthocyanins);
MS-IT detection: The ion trap mass analyzer was used in the full-scan **positive** (anthocyanins) and **negative** ion modes (flavonoids, phenolic acid) , in the *m/z* range 100-1000. MS^2 spectra were automatically performed with helium as the collision gas, using the SmartFrag function.

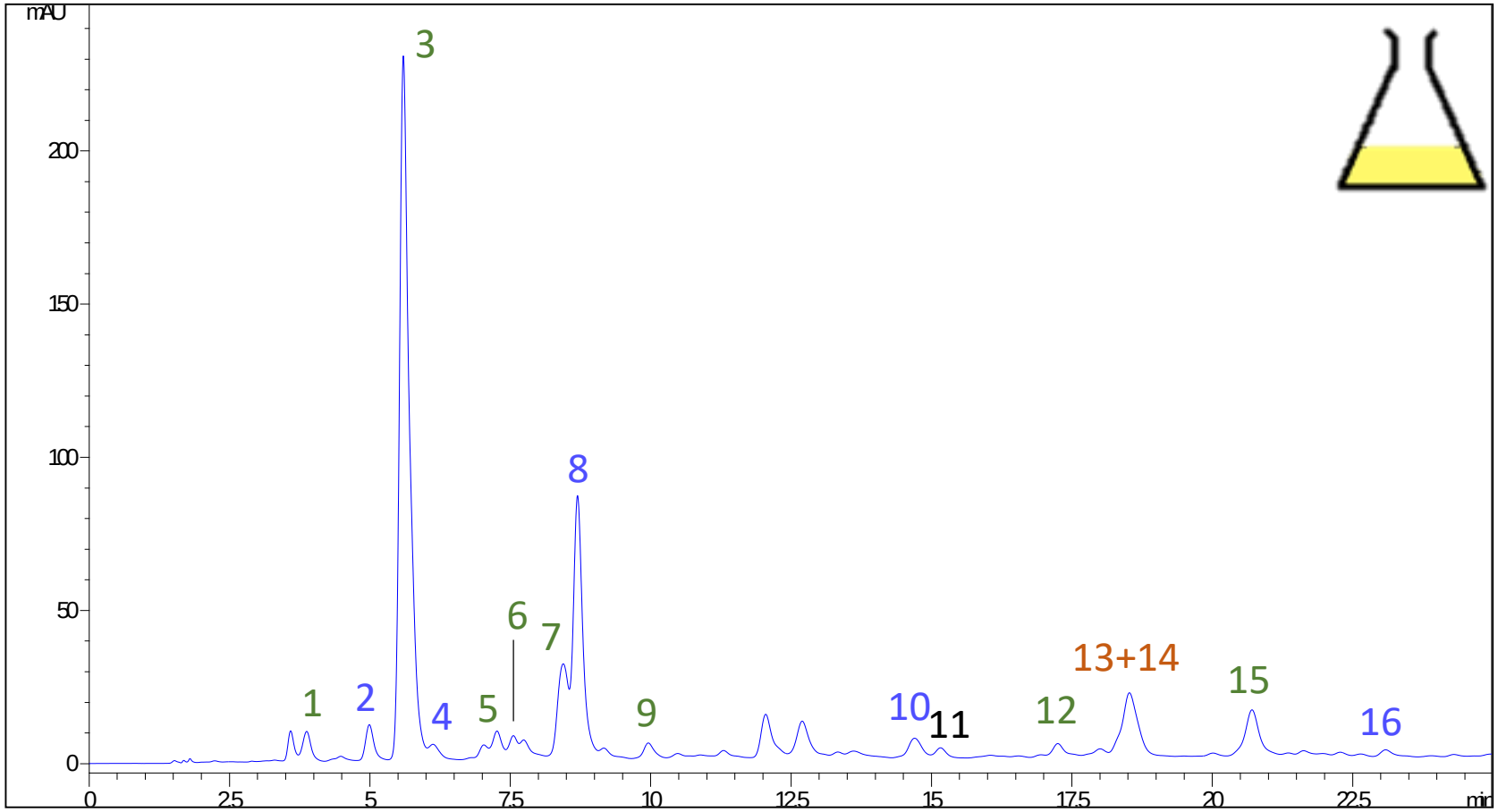
Compounds identification



Peak n°	Anthocyanin
1	Cyanidin-3-O-glucoside
2	Cyanidin-3-O-rutinoside
3	Pelargonidin-3-O-rutinoside
4	Peonidin-3-O-rutinoside

Representative HPLC-UV/DAD chromatogram of **sweet cherry fruit extract** recorded at 520 nm

Compounds identification



Representative HPLC-UV/DAD chromatogram of **sweet cherry fruit extract** recorded at 280 nm

Peak n°	Compound
1	Neochlorogenic acid
2	Catechin
3	3-Coumaroylquinic acid
4	Procyanidin dimer B type
5	5-Caffeoylquinic acid
6	Ferruoyilquinic acid
7	cis-4-Coumaroylquinic acid
8	Epicatechin
9	trans-4-Coumaroylquinic acid
10	Procyanidin dimer B type
11	Naringenin-hexoside
12	3,5-Dicaffeoylquinic acid
13	Quercetin-3-O-glucoside
14	Quercetin-3-O-rutinoside
15	3-Coumaroyl-5-caffeoylquinic acid
16	Catechin-hexoside

Hydroxycinnamic acids Flavan-3-ols Flavonols Other Flavonoids

Come to the unconventional side!

Conventional techniques

- High costs
- Time consuming sample pre-treatment
- Large sample size
- Large solvents and reagents usage

Electrochemical analysis

- Low costs
- Fast (minimal sample pre-treatment)
- Small sample size
- Limited solvents and reagents usage

Amperometric sensors

Amperometric sensors

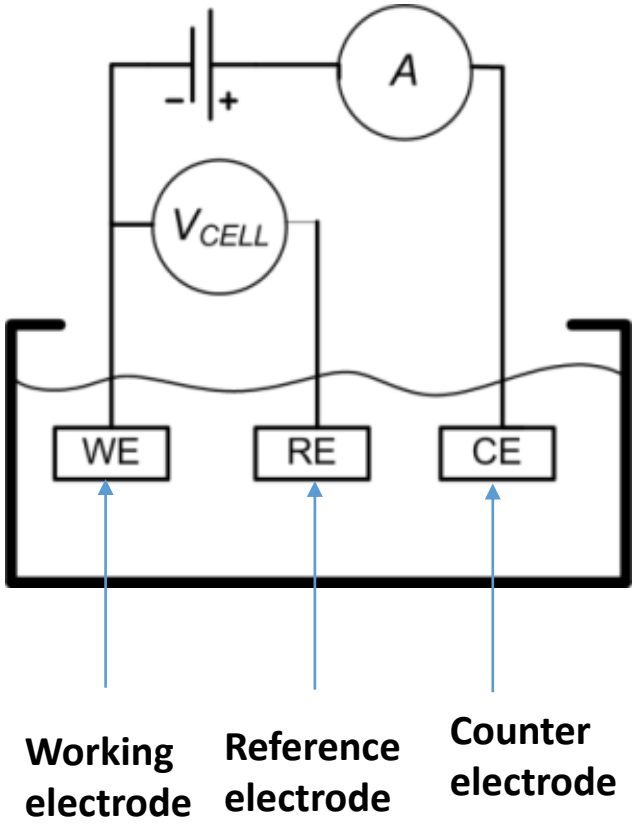


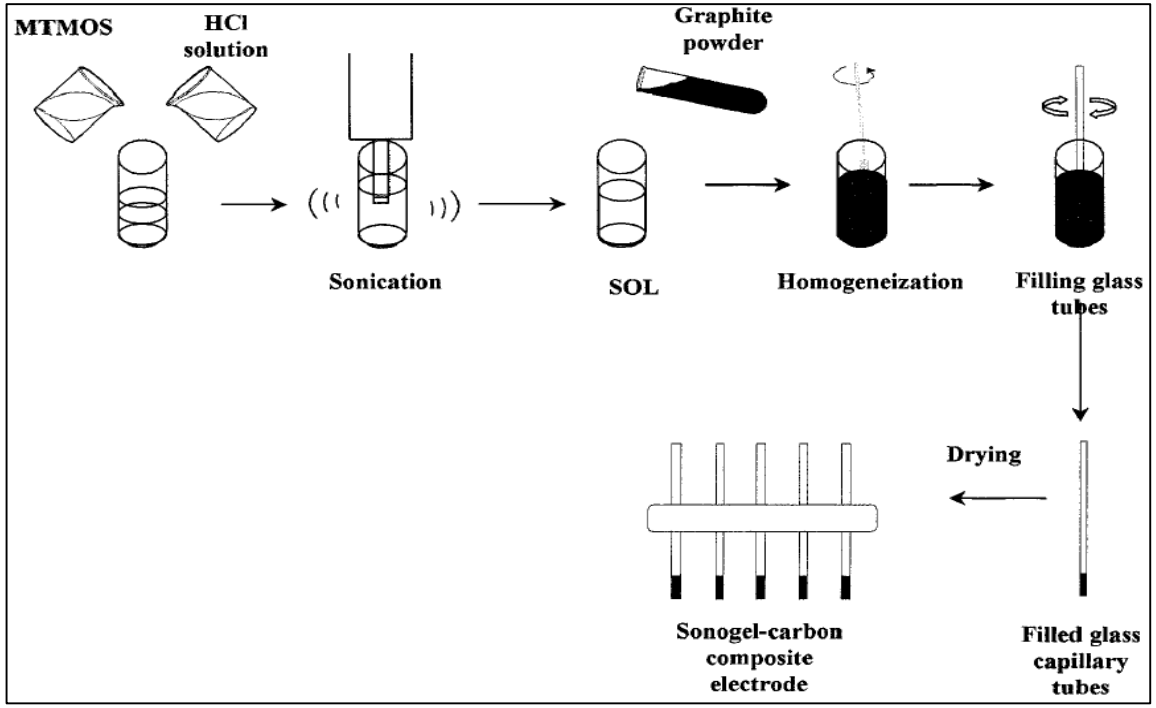
Figure. Scheme of a typical circuit used with amperometric sensors.

- **Chemical sensors**
- measure **current response** to detect the **concentration** of an analyte at a fixed potential.
- The **current** is generated by a **RedOx process** between the **sensor surface** and the **analyte.**
- **RedOX reaction** occurs at the **working electrode**, (the real **sensor**) and determines the passage of current which, in particular conditions, is **proportional** to the **analyte concentration**.

In-line measurement
no sample prep
user-friendly
miniaturization

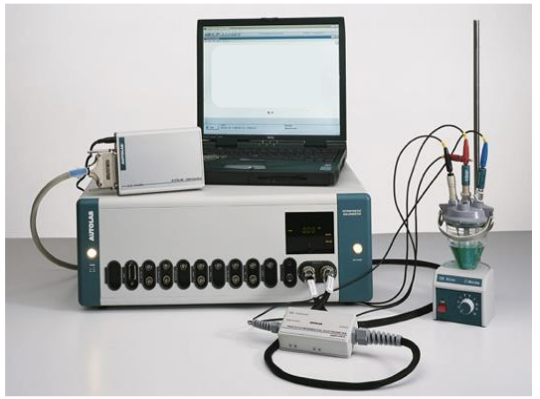
Electrochemical conditions

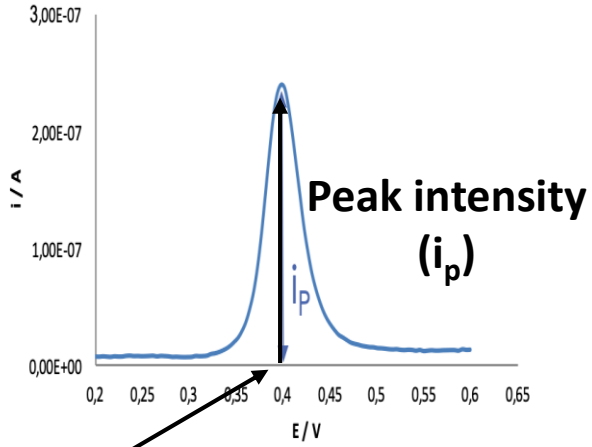
- **Working electrode:** Sonogel Carbon electrode containing Carbon Black (SNGC_CB);
- **Reference electrode:** saturated Ag/AgCl;
- **Auxiliary electrode:** glassy carbon;
- **Working solution:** 0.1 M tartrate buffer solution (TBS), pH= 3.5;



- **Differential Pulse Voltammetry (DPV) parameters:**

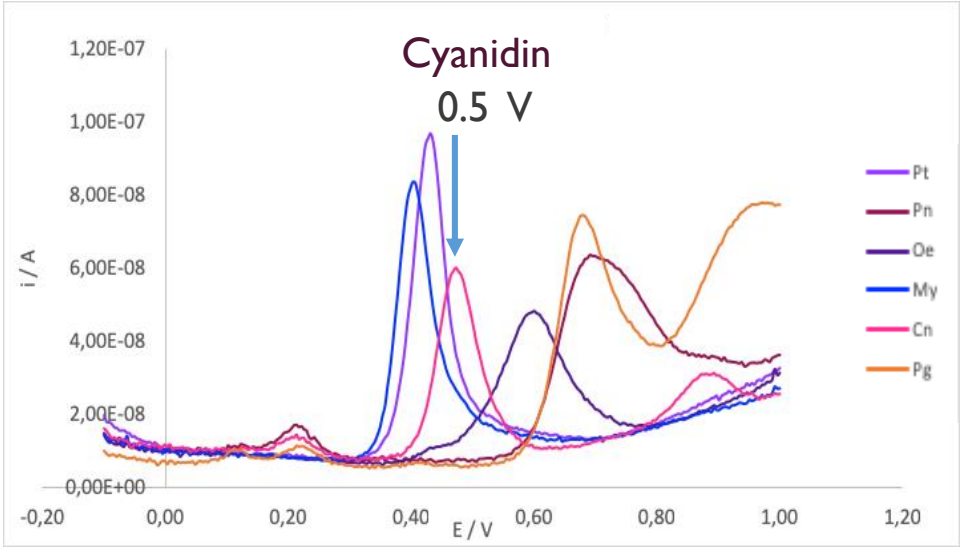
  Pulse time: 0.15 s
  Voltage steptime: 0.6 s
  Modulation amplitude: 0.01 V
  Equilibration time: 5 s



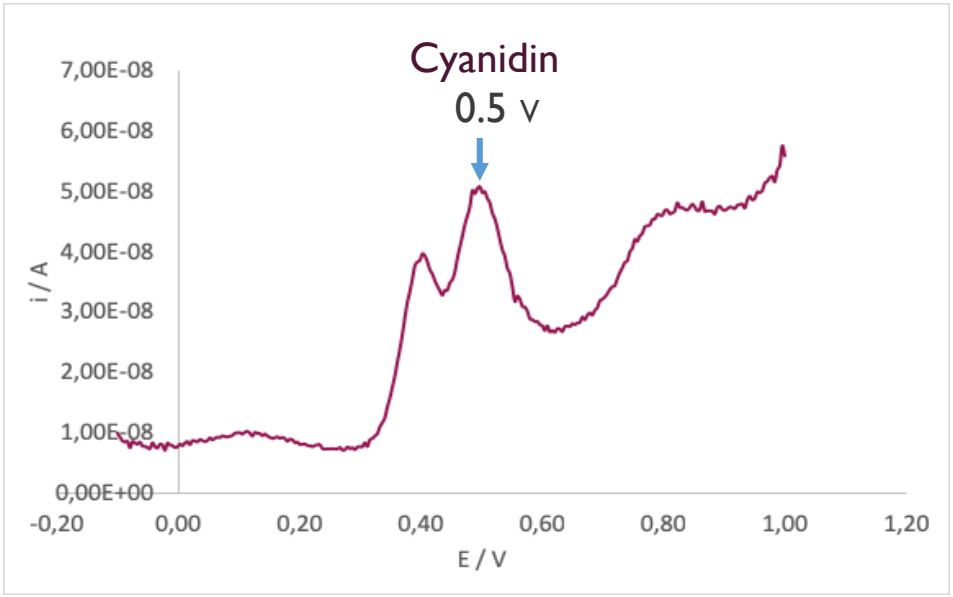


Qualitative analysis of anthocyanins

	compound	E / V
pelargonidin →	Pg	0.683
petunidin →	Pt	0.441
	Ptg	0.432
peonidin →	Pn	0.706
	Png	0.695
delfinidin →	Dpr	0.416
	My	0.407
malvidin →	Mvg	0.613
	Oe	0.601
cyanidin →	Cn	0.473
	Ku	0.487
	Cy	0.508



Anthocyanins reference standard solution



Mashed sweet cherry fruit

Summary

1. Background and aim

2. Analytical characterisation of sweet-cherry fruit extracts

3. ***In vivo*** **assessment of the anti-oxidant activity on *C. elegans* model**

In collaboration with the research group of Prof. Celestino Santos-Buelga of the University of Salamanca

Workflow

Sweet cherry fruit («Moretta» variety)

Preparation of the extract

In vivo assays on *C. elegans*

Resistance to thermal stress

Preparation of the extract

«Moretta» sweet cherry fruit

Dynamic maceration
MeOH/2% HCl
95:5 (v/v)

Concentration

Purification with water on C_{18} column

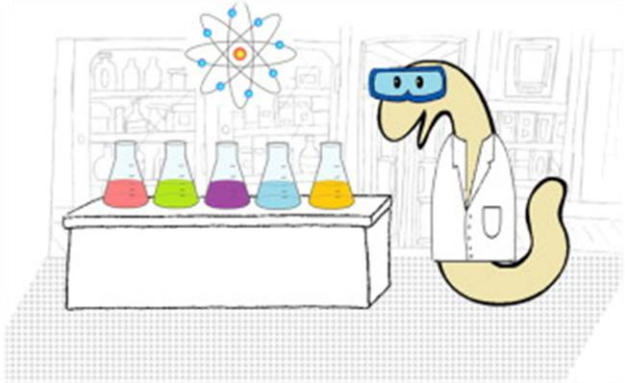
Lyophilization

Final Cherry Extract (CE)

Experimental

- The effects on the **resistance to thermal stress** of the extract obtained from “Moretta” cherries have been evaluated.

- Thermal stress assays were carried out in **wild type N2** of the model nematode *C. elegans* **grown** on NGM agar plates **in the presence and absence** (control group) of the **cherry extract** at different concentrations, on the basis of previous experiments.



- Resistance to thermal stress was evaluated by measuring the **rate of survival**, through the response to a mechanical stimulus, after **6 or 8 hours** of **exposure** to the **lethal temperature of 35 °C** at different stages of the development (2^{nd} and 9^{th} day of adulthood).

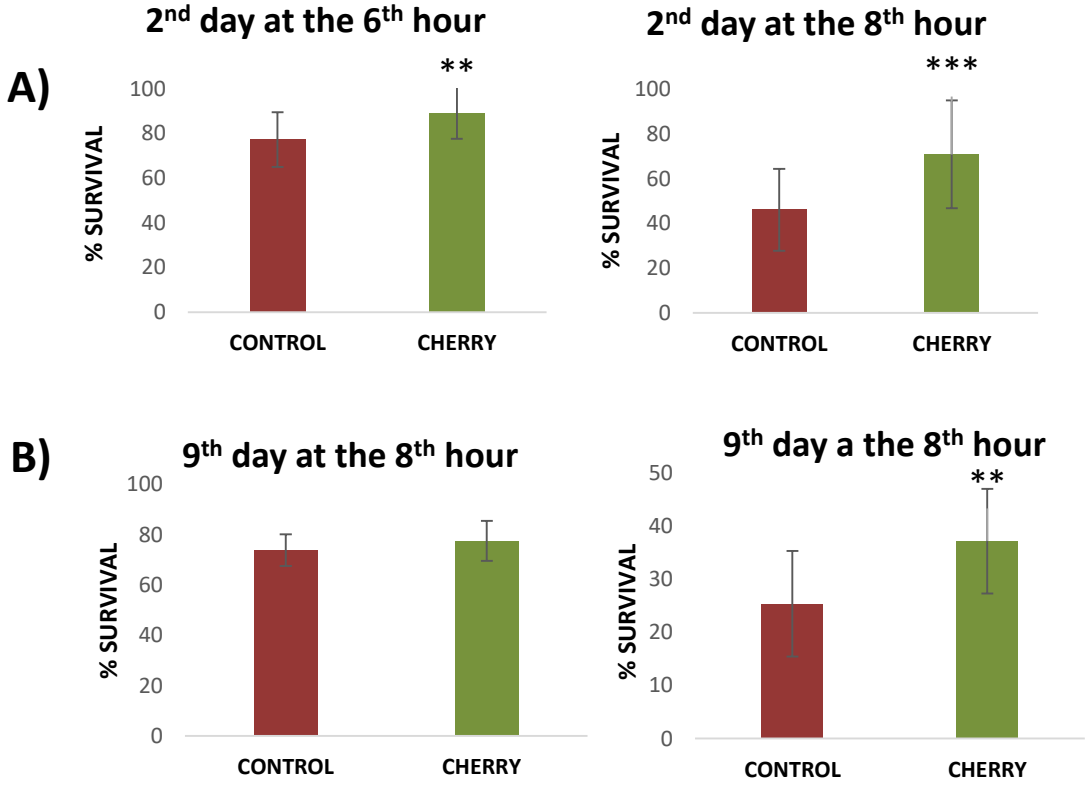
- Assays were performed with approximately 100 nematodes for treatment, and each trial was made in triplicate. In each assay, the results were expressed as percentage of living animals.

Results

- **None** of the concentration of the cherry extract tested leaded to **toxic effect** on nematodes.

- The most significant results on the anti-oxidant activity were obtained at a concentration of 25 µg/mL. Indeed, an **increase in survival** in the nematodes treated with cherry extract both on the **second and ninth day** of worm life was evident.

- The most significant result was obtained on the **second day** (**A**) for a thermal stress of **eight hours** where there was a 25% increase in the survival of treated worms compared to the control. The differences were considered significant at *(p<0,05), **(p<0,01) and ***(p<0,001).



Overall conclusions

- In this work, cherry fruit belonging to the «Moretta» variety were characterized in the composition of their polyphenolic fraction by means of conventional analytical techniques. The **polyphenolic fraction** of «Moretta» cherry fruit encompassed **anthocyanins**, **hydroxycinnamic acids, flavan-3-ols** and other flavonoids.

- An **innovative analytical tool** based on electrochemical analysis with **SNGC_CB amperometric sensors** for the **fast** determination of anthocyanins in sweet cherry fruit was developed. The sensor proved capable of selectively identify the major class of anthocyanins in cherry fruit, namely cyanidin-3-*O*-glycosides. Future steps will include the quantitative determination of the anthocyanins by using this innovative technique.

- Given the high biological value of the polyphenolic fraction of “Moretta” cherry fruit, its extract was submitted to *in vivo* antioxidant activity assays by using the *C. elegans* model. The extract was able **to significantly increase the adult worm resistance to oxidative and thermal stress** at a concentration of **25 µg/mL**.

- Moretta cherry extract represents a **promising source** of bioactive compounds, thus it might find an application in the nutraceutical industry.

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Thank you!!

…questions?

e-mail contact:

virginia.brighenti@unimore.it