



# Identificazione e quantificazione di Polifenoli da mirtillo (integratore alimentare) mediante estrazione in fase solida (SPE)

Esercitazione  
16 Marzo 2023



# Attività biologica

- Nelle piante: meccanismo di difesa
  - Stress abiotico → UV radiations
  - Stress biotico →
    - parassiti
    - agenti patogeni

- Attività antiossidante



Prevenzione del danno ossidativo delle biomolecole (DNA, lipidi e proteine)



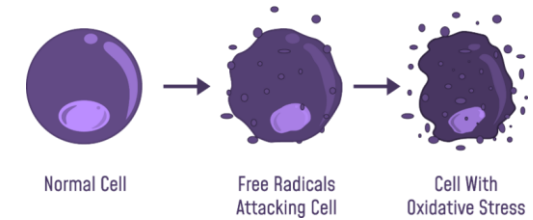
Protegge dalle specie reattive dell'ossigeno (ROS)



Benefici per la salute: proprietà antinfiammatorie, antimicrobiche, neuroprotettive, cardioprotettive, antivirali, antitumorali

- Conservanti naturali per alimenti → Prevenire il deterioramento ossidativo e la contaminazione microbica

## OXIDATIVE STRESS



- Superoxide anion ( $\cdot\text{O}_2^-$ )
- Hydroxyl radical ( $\cdot\text{OH}$ )
- Nitric oxide ( $\text{NO}\cdot$ )
- Peroxynitrite anion ( $\text{ONOO}^-$ )

**Stress ossidativo**

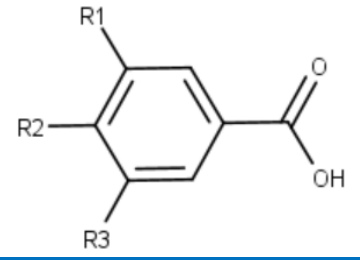


- disturbi del metabolismo
- malattie cardiovascolari
  - cancro
  - obesità
  - diabete

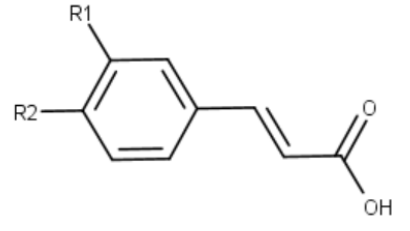
# Composti fenolici

## Phenolic Acids C<sub>6</sub>-C<sub>1</sub>

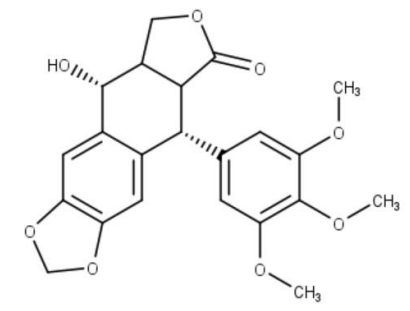
### Hydroxybenzoic Acid



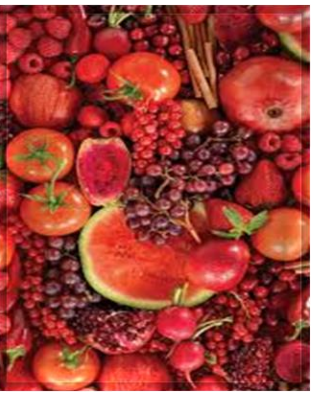
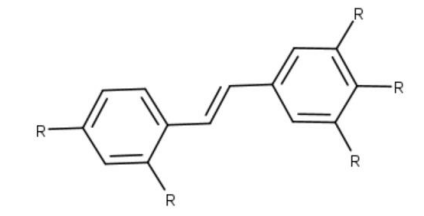
### Hydroxycinnamic Acid



## Lignans C<sub>22</sub>H<sub>22</sub>O<sub>8</sub>

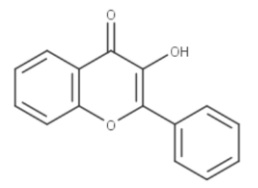


## Stilbenes C<sub>14</sub>H<sub>12</sub>

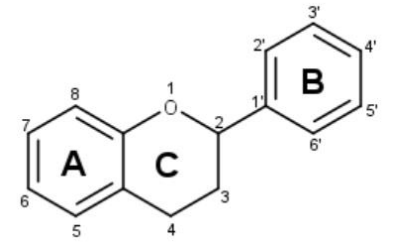
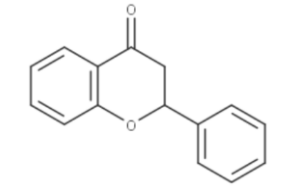


## Flavanoids C<sub>6</sub>-C<sub>3</sub>-C<sub>6</sub>

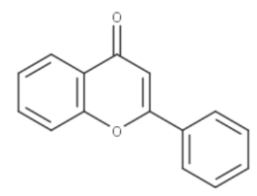
### Flavonols C<sub>15</sub>H<sub>10</sub>O<sub>3</sub>



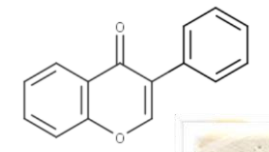
### Flavanones C<sub>15</sub>H<sub>12</sub>O<sub>2</sub>



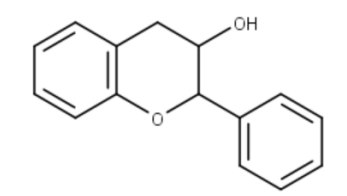
### Flavones C<sub>15</sub>H<sub>10</sub>O<sub>2</sub>



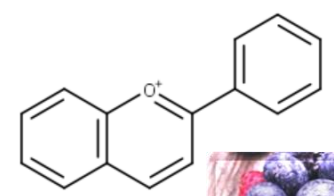
### Isoflavones C<sub>15</sub>H<sub>10</sub>O<sub>2</sub>



### Flavan-3-ols C<sub>15</sub>H<sub>14</sub>O<sub>2</sub> (Catechines and Proantocyanidines)

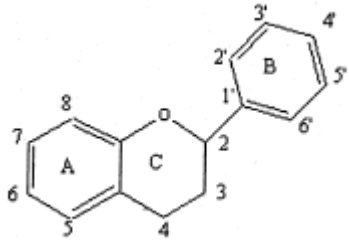


### Anthocyanidin C<sub>15</sub>H<sub>11</sub>O<sup>+</sup>



# Derivativi dei composti fenolici

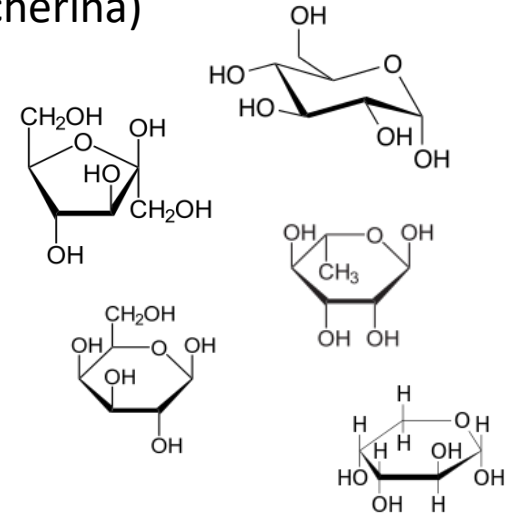
**Aglicone** (parte non zuccherina)



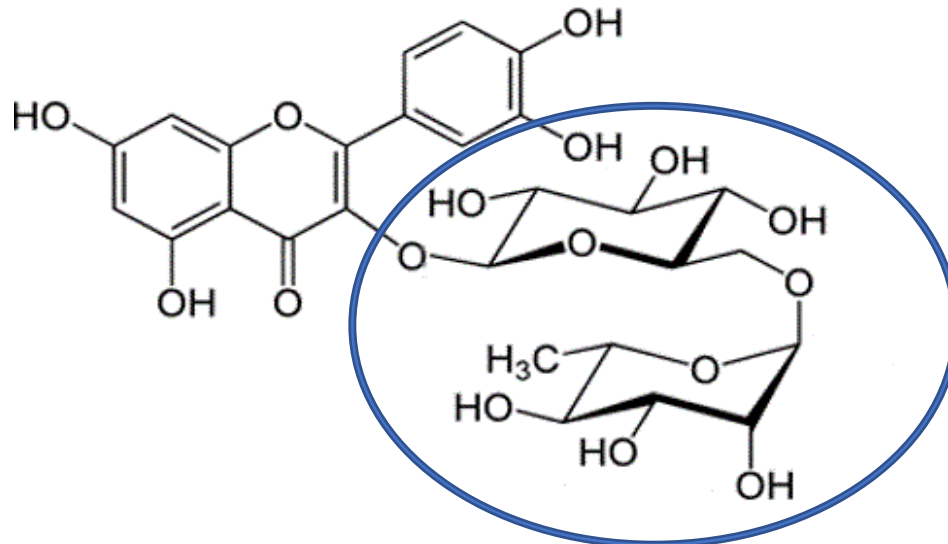
Anello benzenico (A) condensato con anello a sei termini (C) anello piranico e in posizione 2 ha un anello fenilico (B) come sostituente.

**Glicone** (parte zuccherina)

- **glucosides** (glycone = glucose)
- **fructosides** (glycone = fructose)
- **ramnosides** (glycone = rhamnose)
- **galactosides** (glycone = galactose)
- **arabinosides** (glycone = arabinose)
- ....etc

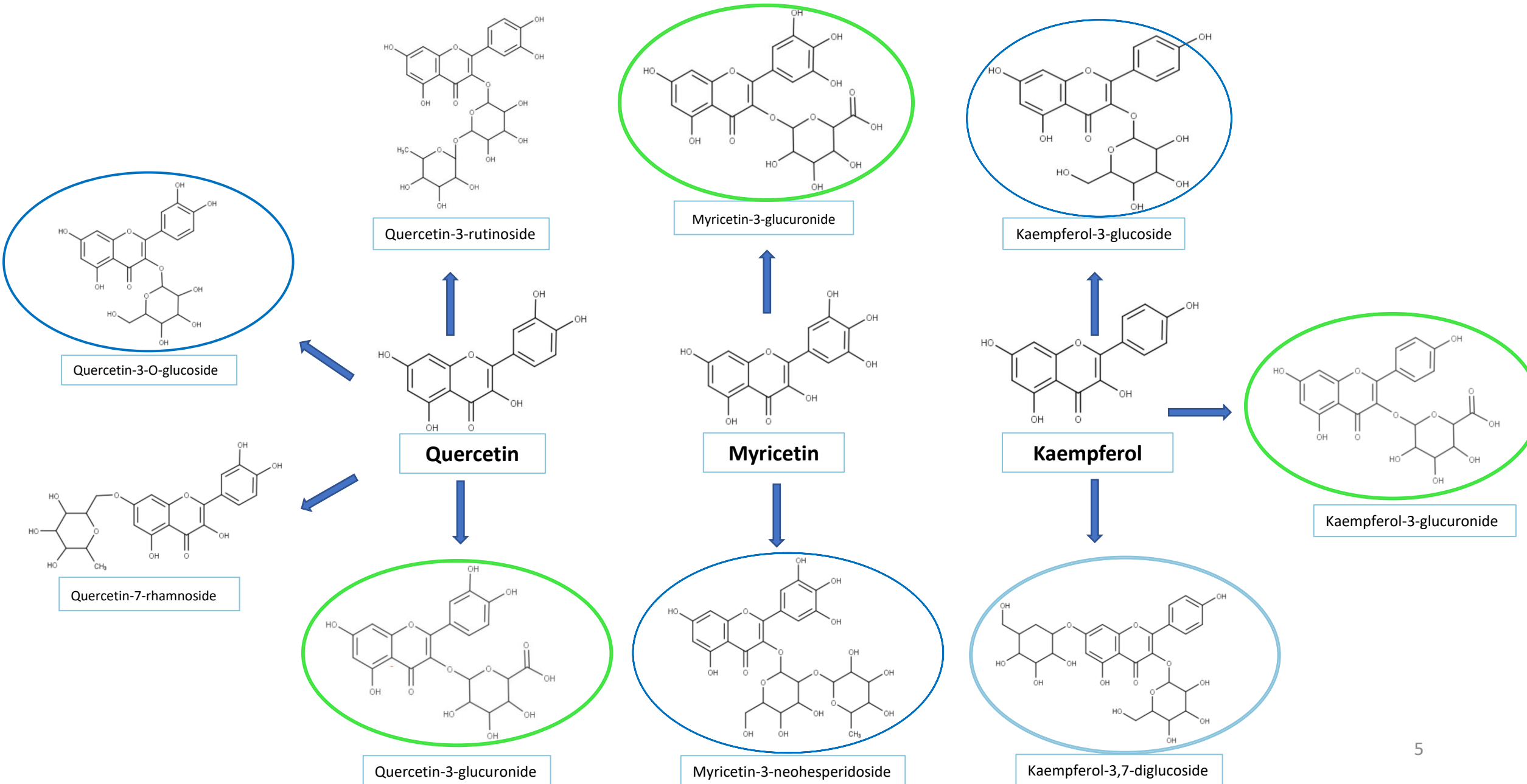


**Aglicone:**  
**Quercetina**



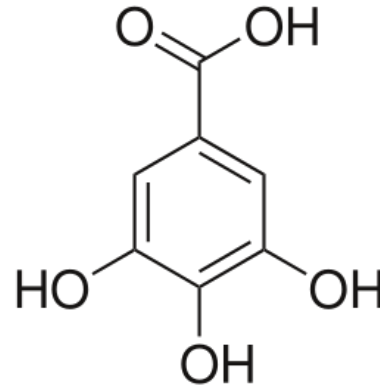
**Glicone:**  
**Rutinosio**

# Flavonoidi

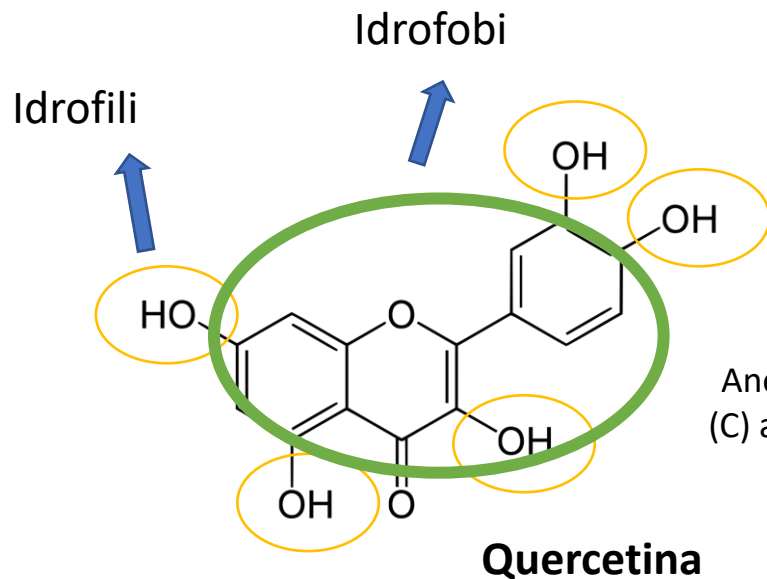


# Caratteristiche dei composti fenolici

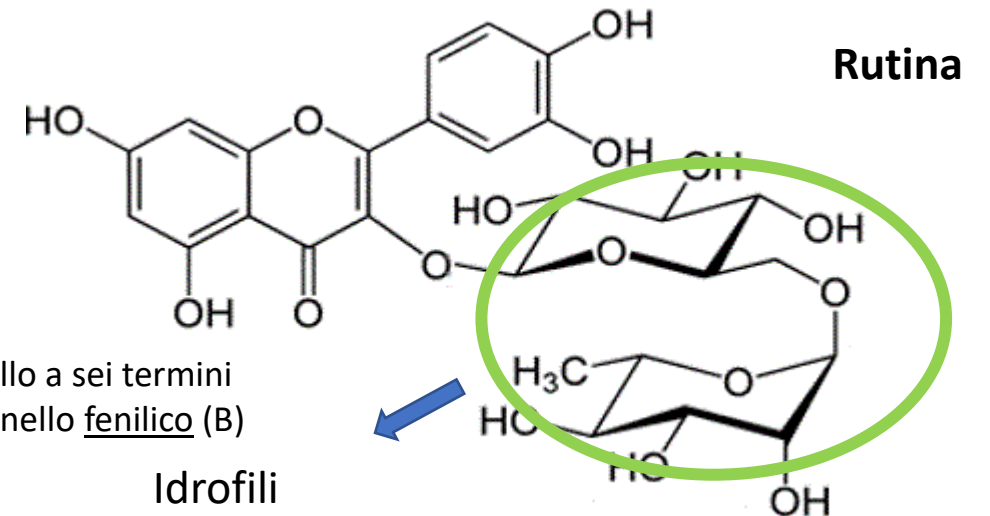
| Gruppo funzionale  | Nome                | Descrizione   |
|--|---------------------|---|
| $-OH$  | ossidrilico - OH    | polare e solubile in acqua: tende a formare legami a H            |
| $\begin{array}{c} O \\ // \\ -C \\   \\ OH \end{array}$    | carbossilico - COOH | acido debole: tende alla forma $-COO^-$ perdendo uno ione $H^+$   |
| $\begin{array}{c} H \\   \\ -N \\   \\ H \end{array}$      | amminico - $NH_2$   | base debole: tende alla forma $NH_3^+$ acquistando uno ione $H^+$ |
| $\begin{array}{c} H \\   \\ -C=O \end{array}$              | aldeidico - COH     | polare e solubile in acqua  |
| $>C=O$   | chetonico =CO       | polare e solubile in acqua  |
| $\begin{array}{c} O \\    \\ -P-OH \\   \\ OH \end{array}$ | fosfato - $PO_3H_2$ | acido: tende alla forma $PO_3^{2-}$ perdendo due $H^+$            |



**Acido Gallico**



Anello benzenico (A) condensato con anello a sei termini (C) anello piranico e in posizione 2 ha un anello fenilico (B) come sostituente.



**Idrofili**

# Solventi

## Polarity of Solvents

Water  
Acetic Acid  
Ethylene glycol  
Methanol  
Ethanol  
Isopropanol  
Pyridine  
Acetonitrile  
Nitromethane  
Diethylamine  
Aniline  
Dimethylsulfoxide  
Ethylacetate  
Dioxane  
Acetone  
Dichloroethane  
Tetrahydrofuran  
Dichloromethane  
Chloroform  
Diethylether  
Benzene  
Toluene  
Xylene  
Carbontetrachloride  
Cyclohexane  
Petroleum ether  
Hexane  
Pentane



Polar

Non-polar

**Polar Solvents**

**DMF**

**DMSO**

**Water**

**Acetone**

**Methanol**

**Isopropanol**

**Acetonitrile**

**Non-Polar Solvents**

**Alkanes**

**Benzene**

**Toluene**

**Acetic acid**

**Chloroform**

**Diethyl ether**

**Ethyl acetate**

# Campione in esame



**Integratore alimentare su base di mirtillo rosso**



# Estrazione

Polvere di mirtillo



Pesare 1g di polvere di mirtillo



5 mL di 70:30 MeOH:H<sub>2</sub>O



3 minuti di Vortex



5 mL di 70:30 MeOH:H<sub>2</sub>O



Analisi LC-MS/MS



Diluzione 1:10



- ✓ Attivazione: 1 mL di MeOH
- ✓ Condizionamento : 1 mL di H<sub>2</sub>O:MeOH 90:10 (H<sub>2</sub>O)
- ✓ Carico: campione diluito 1:100 in 1 mL of H<sub>2</sub>O:MeOH 90:10 (H<sub>2</sub>O)
- ✓ Lavaggio: 1 mL di H<sub>2</sub>O
- ✓ Eluzione:
- ✓ 1) 1mL di H<sub>2</sub>O
- ✓ 2) 1mL di MeOH
- ✓ 3) 1mL di ACN



Clean-Up



5000 rpm per 15 minuti

# Parametri cromatografia liquida accoppiata alla spettrometria di massa (LC-MS/MS)



**ACE C18-PFP**



**Pentafluorophenyl (PFP)**

**LC System:** Shimadzu Nexera XR LC 20 AD system

**Colonna:** ACE Excel 2 C18-PFP 2.0  $\mu\text{m}$  100x2.1 mm

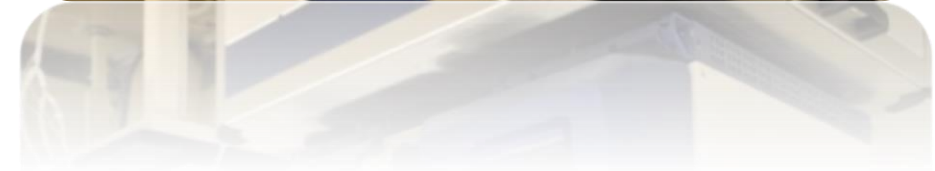
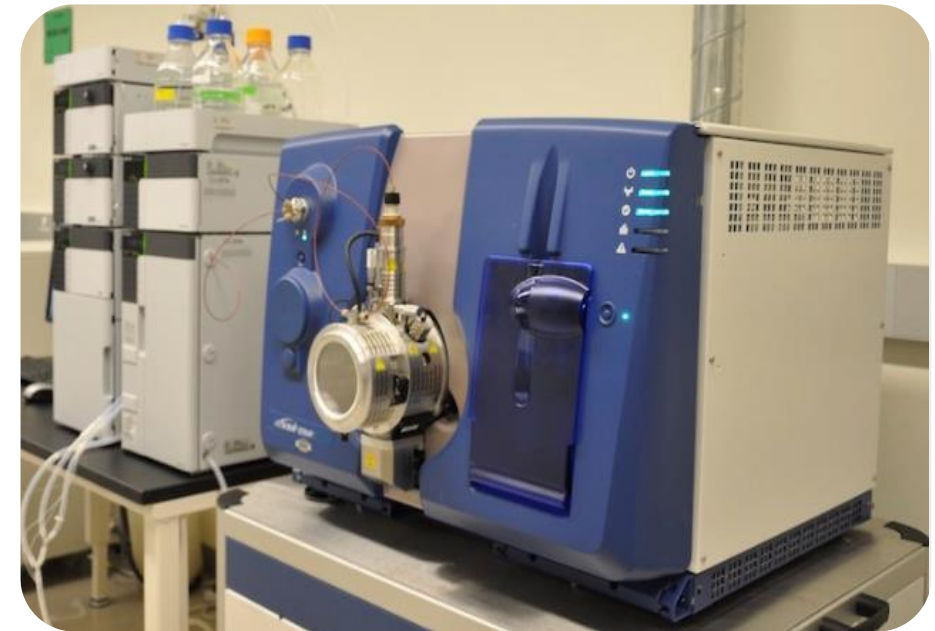
**Flusso:** 0.4 mL/min

**Fase A:** H<sub>2</sub>O 1% CH<sub>3</sub>COOH

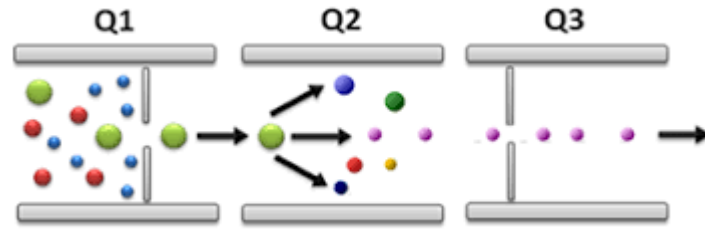
**Fase B:** ACN

**MS/MS system:** Sciex Qtrap4500

**Ionizzazione:** ESI (-)



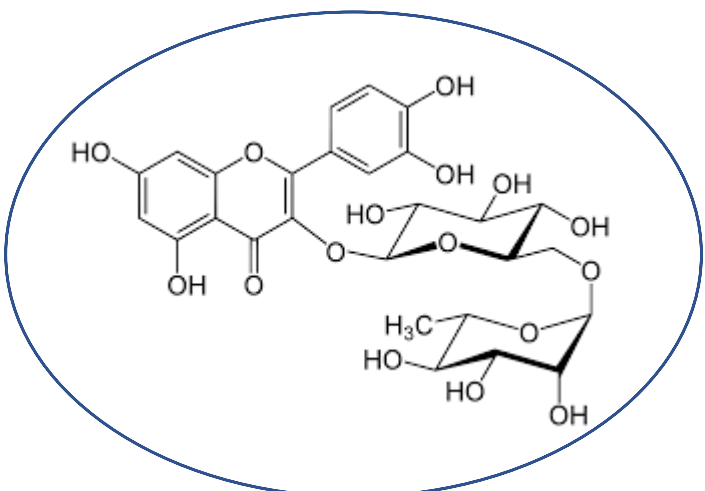
# Modalità di acquisizione: Multiple Reaction Monitoring (MRM)



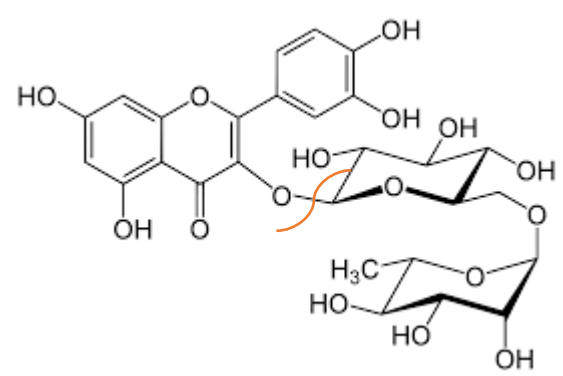
**Q1:**  
Selezione ione precursore

**Q2:**  
Lo ione precursore viene frammentato nella cella di collisione

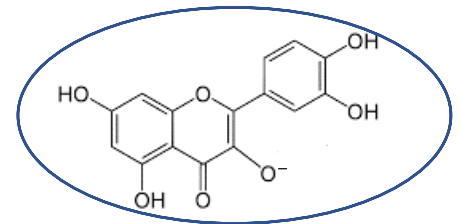
**Q3:**  
Selezione dello ione prodotto



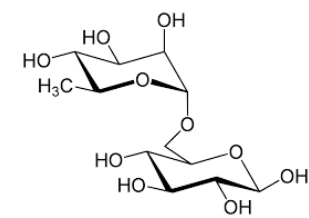
**Q1**  
Parent ion



**Q2**



**Aglicone**



**Glicone**

**Q3**  
daughter ion

300.9 m/z

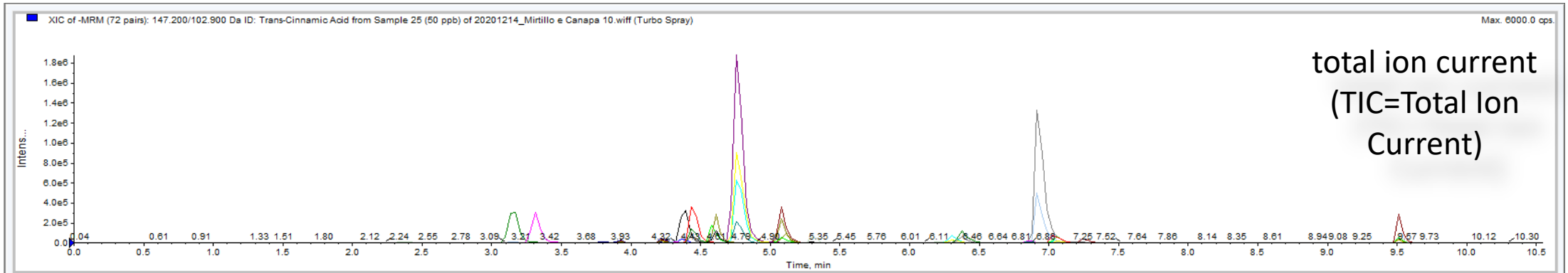
# Mass spectrometry parameters:

## Infusione dello standard in MS/MS

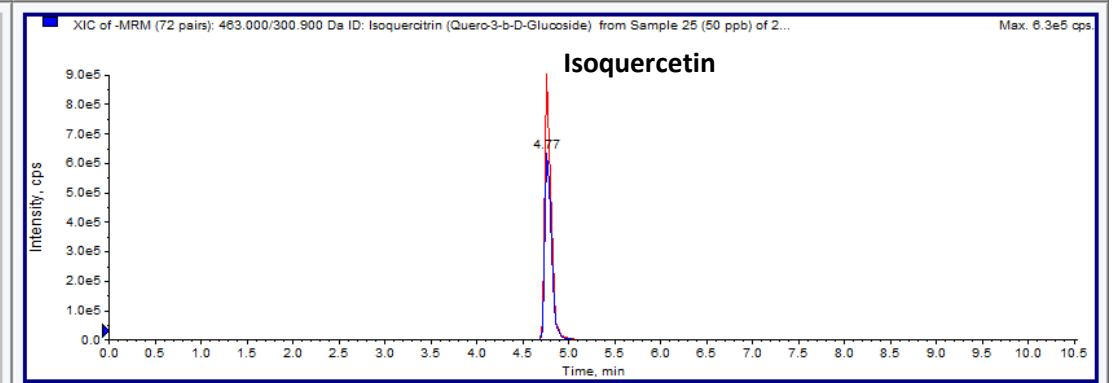
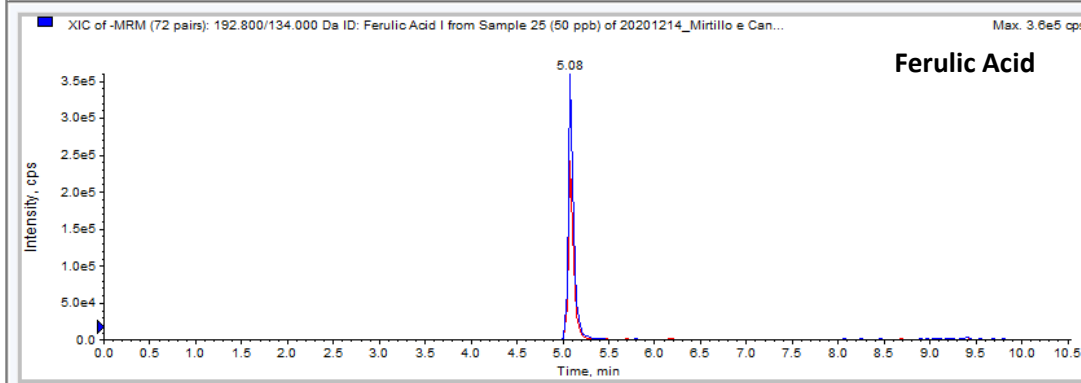
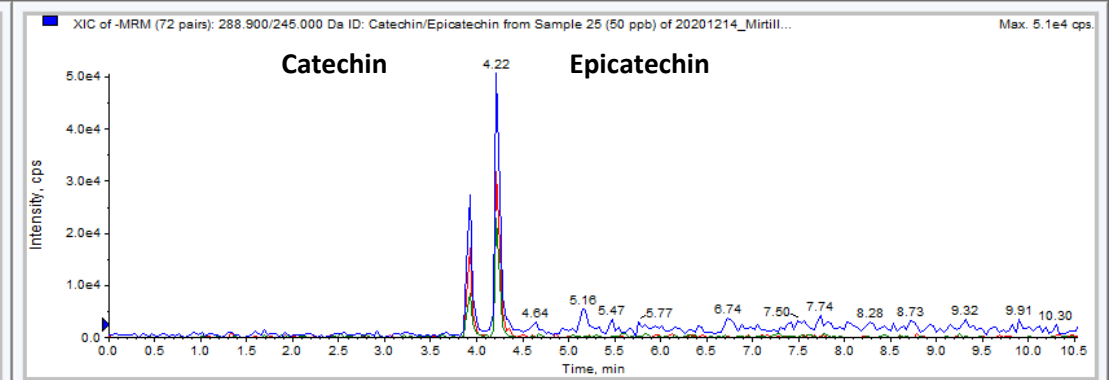
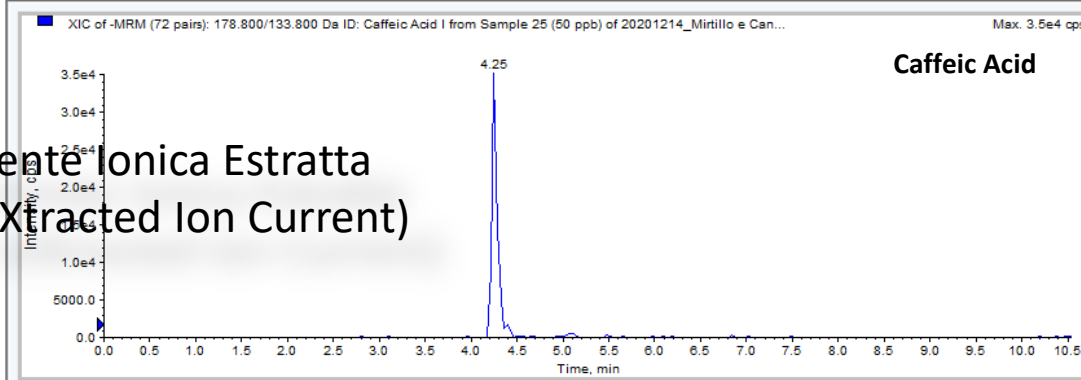
| ID                   | Q1    | Dp     | Ep    | Q3            | CE    | CXP   | ID  | Q1    | Dp     | Ep    | Q3                                  | CE    | CXP   | ID                                 | Q1    | Dp     | Ep    | Q3    | CE    | CXP   |  |
|----------------------|-------|--------|-------|---------------|-------|-------|---|-------|--------|-------|-------------------------------------|-------|-------|------------------------------------|-------|--------|-------|-------|-------|-------|--|
| Epicatechin/Catechin | 288,9 | -85,0  | -8,0  | 245,0         | -24,0 | -9,0  | Vanillic Acid                             | 167,1 | -49,0  | -11,0 | 107,9                               | -28,5 | -5,0  | 3/4-OH-Benzoic Acid                | 136,9 | -12,0  | -9,0  | 89,0  | -15,5 | -6,0  |  |
|                      |       |        |       | 108,9         | -38,5 | -8,0  |   |       |        |       | 151,9                               | -19,5 | -6,5  |                                    |       |        |       | 66,0  | -30,0 | -10,0 |  |
| Ferulic Acid         | 192,8 | -63,0  | -11,0 | 134,0         | -21,0 | -8,0  | Apigenin                                  | 268,9 | -110,0 | -4,0  | 116,9                               | -50,0 | -9,0  | Trans-Cinnamic Acid                | 147,2 | -50,0  | -8,0  | 102,9 | -16,0 | -4,0  |  |
|                      |       |        |       | 177,8         | -18,0 | -6,0  |   |       |        |       | 151,0                               | -34,0 | -5,0  |                                    |       |        |       | 77,2  | -30,0 | -6,0  |  |
| Gallic Acid          | 168,9 | -70,0  | -10,5 | 124,9         | -21,0 | -9,0  | Myricetin                                 | 316,9 | -115,0 | -5,0  | 150,9                               | -34,0 | -6,0  | Orientin (Luteolin-8-Glucoside)    | 447,1 | -105,0 | -11,0 | 356,9 | -29,0 | -11,0 |  |
|                      |       |        |       | 96,9          | -26,0 | -6,0  |   |       |        |       | 136,9                               | -36,0 | -9,5  |                                    |       |        |       | 297,0 | -43,0 | -11,0 |  |
| o-Coumaric Acid      | 163,0 | -50,0  | -5,0  | 119,0         | -19,0 | -8,0  | Luteolin                                  | 284,9 | -100,0 | -8,0  | 150,9                               | -35,0 | -11,0 | Hyperoside (Querc-3-D-Galactoside) | 462,9 | -110,0 | -5,0  | 300,9 | -48,0 | -10,0 |  |
|                      |       |        |       | 117,0         | -33,0 | -7,0  |   |       |        |       | 199,0                               | -35,0 | -6,0  |                                    |       |        |       | 299,9 | -40,0 | -11,0 |  |
| p-Coumaric Acid      | 162,8 | -60,0  | -5,0  | 119,0         | -20,0 | -9,0  | Trans 3OH Cinnamic Acid (m-coumaric acid) | 163,1 | -50,0  | -6,0  | 118,9                               | -19,0 | -9,0  | Tyrosol                            | 137,0 | -65,0  | -9,0  | 119,0 | -21,0 | -8,5  |  |
|                      |       |        |       | 116,7         | -42,0 | -9,0  |   |       |        |       | 117,0                               | -33,0 | -7,0  |                                    |       |        |       | 106,9 | -23,0 | -5,5  |  |
| Quercetin            | 300,9 | -94,0  | -10,0 | 151,0         | -30,0 | -6,0  | Kampferol                                 | 285,0 | -135,0 | -7,0  | 228,8                               | -40,0 | -9,0  | (-)-Epigallocatechin               | 305,0 | -98,0  | -6,5  | 136,9 | -36,0 | -10,0 |  |
|                      |       |        |       | 178,8         | -26,0 | -6,0  |   |       |        |       | 159,0                               | -42,0 | -6,0  |                                    |       |        |       | 166,9 | -28,5 | -6,5  |  |
| Caffeic Acid         | 178,8 | -60,0  | -10,0 | 133,8         | -33,0 | -7,0  | Isoquercitrin (Querc-3-b-D-Glucoside)     | 463,0 | -100,0 | -9,0  | 300,9                               | -33,0 | -11,0 | (-)-Epigallocatechin Gallato       | 457,2 | -10,0  | -7,0  | 168,8 | -20,0 | -17,0 |  |
| Rosmarinic Acid      | 359,0 | -78,0  | -4,5  | 160,8         | -24,0 | -7,0  |   |       |        |       | 270,9                               | -58,0 | -10,0 |                                    |       |        |       | 124,9 | -40,0 | -17,0 |  |
| Rutin                | 609,2 | -100,0 | -6,0  | 300,9         | -45,0 | -11,0 | Chlorogenic Acid                          | 353,1 | -60,0  | -7,0  | 190,9                               | -25,5 | -6,5  | OH-Tyrosol                         | 152,9 | -75,0  | -7,0  | 122,9 | -22,0 | -8,0  |  |
|                      |       |        |       | 254,7         | -70,0 | -9,0  |   |       |        |       | 160,9                               | -35,0 | -6,0  |                                    |       |        |       | 104,6 | -30,0 | -7,0  |  |
|                      |       |        |       | Siringic Acid | 196,9 | -57,0 | -11,0                                     | 181,9 | -19,0  | -6,0  | Diosmetin (Luteolin-4-methyl ether) | 299,1 | -90,0 | -6,0                               | 255,8 | -40,0  | -9,0  |       |       |       |  |
|                      |       |        |       | 120,9         | -22,0 | -8,0  | Oleuropein                                | 539,2 | -55,0  | -10,0 | 275,0                               | -32,0 | -9,0  |                                    |       |        |       |       |       |       |  |

- **Declustering Potential (DP):** Il parametro DP controlla il voltaggio applicato all'orifizio che controlla la capacità di separare i cluster di ioni tra l'orifizio e la guida ionica QJet . È impiegato per ridurre al minimo i cluster di solvente che possono restare sugli ioni campione dopo che questi entrano nella camera del vuoto e, se necessario, per frammentare gli ioni. Maggiore sarà la tensione, maggiore sarà l'energia impartita agli ioni. Se il parametro è impostato su un valore troppo alto, potrebbe verificarsi una frammentazione indesiderata.
- **Entrance Potential (EP):** Il parametro EP è il potenziale in entrata che guida e focalizza gli ioni attraverso l'alta pressione applicata alla regione Q0.
- **Collision Energy (CE):** Il parametro CE controlla la differenza di potenziale tra la regione Q0 e la camera di collisione Q2. È usato solo nelle scansioni di tipo MS/MS. Questo parametro corrisponde alla quantità di energia che gli ioni precursori ricevono quando sono accelerati nella camera di collisione Q2, dove collidono con le molecole di gas e i frammenti.
- **Collision Cell Exit Potential (CXP):** Il parametro CXP è impiegato solo nelle scansioni di tipo Q3 e MS/MS. Questo parametro trasmette gli ioni nel quadrupolo Q3.

# Analisi qualitativa di composti fenolici



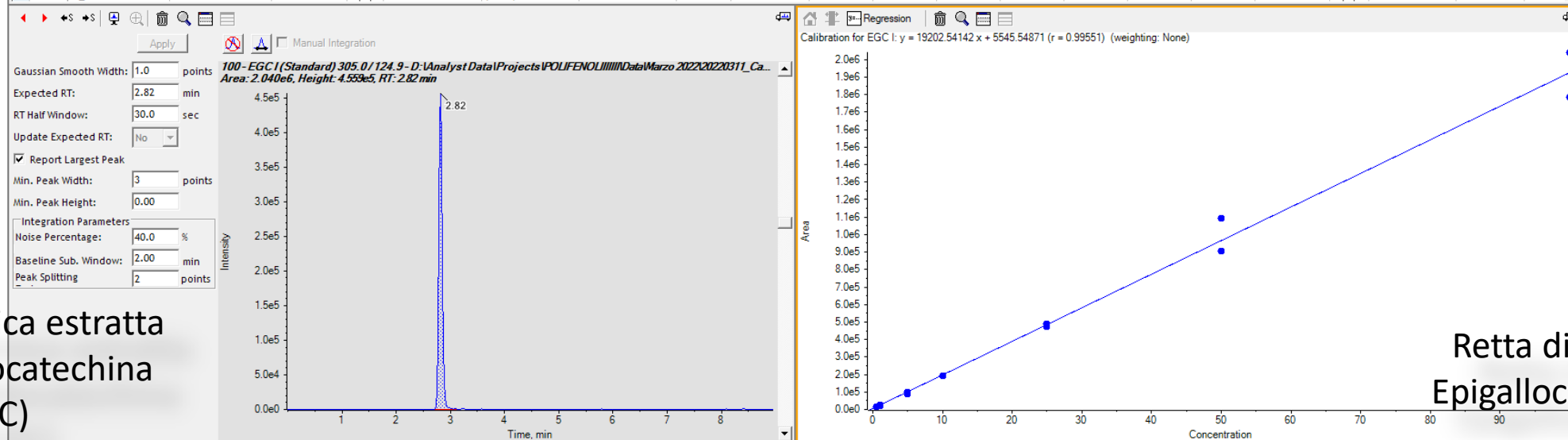
Corrente Ionica Estratta  
(XIC=EXtracted Ion Current)



# Analisi quantitativa di composti fenolici

Retta + Set di campioni  
iniezzati

| Index | Sample Name | Sample ID | Sample Type | IS                                  | Component Name | IS Name | Component Group Name | Outlier Reasons | Actual Concentration | Area    | IS Area | Area Ratio | Height  | IS Area / Height | Retention Time | Width at 50% | Signal / Noise | Used                                | Calculated Concentration | Accuracy |
|-------|-------------|-----------|-------------|-------------------------------------|----------------|---------|----------------------|-----------------|----------------------|---------|---------|------------|---------|------------------|----------------|--------------|----------------|-------------------------------------|--------------------------|----------|
| 125   | 1           |           | Standard    | <input type="checkbox"/>            | EGC I          | (No IS) |                      |                 | 1.0000               | 2.497e4 | N/A     | N/A        | 6.020e3 | N/A              | 2.82           | 0.07         | 61.0           | <input checked="" type="checkbox"/> | 1.012e0                  | 101.17   |
| 185   | 1           |           | Standard    | <input type="checkbox"/>            | EGC I          | (No IS) |                      |                 | 1.0000               | 2.194e4 | N/A     | N/A        | 4.137e3 | N/A              | 2.81           | 0.08         | 61.9           | <input checked="" type="checkbox"/> | 8.536e-1                 | 85.36    |
| 245   | 5           |           | Standard    | <input type="checkbox"/>            | EGC I          | (No IS) |                      |                 | 5.0000               | 1.005e5 | N/A     | N/A        | 2.372e4 | N/A              | 2.82           | 0.07         | 271.9          | <input checked="" type="checkbox"/> | 4.946e0                  | 98.92    |
| 305   | 5           |           | Standard    | <input type="checkbox"/>            | EGC I          | (No IS) |                      |                 | 5.0000               | 8.974e4 | N/A     | N/A        | 1.807e4 | N/A              | 2.80           | 0.07         | 273.3          | <input checked="" type="checkbox"/> | 4.385e0                  | 87.69    |
| 365   | 10          |           | Standard    | <input type="checkbox"/>            | EGC I          | (No IS) |                      |                 | 10.0000              | 1.927e5 | N/A     | N/A        | 4.910e4 | N/A              | 2.82           | 0.06         | 367.8          | <input checked="" type="checkbox"/> | 9.746e0                  | 97.46    |
| 425   | 10          |           | Standard    | <input type="checkbox"/>            | EGC I          | (No IS) |                      |                 | 10.0000              | 1.916e5 | N/A     | N/A        | 3.853e4 | N/A              | 2.81           | 0.08         | 488.9          | <input checked="" type="checkbox"/> | 9.690e0                  | 96.90    |
| 485   | 25          |           | Standard    | <input type="checkbox"/>            | EGC I          | (No IS) |                      |                 | 25.0000              | 4.887e5 | N/A     | N/A        | 1.195e5 | N/A              | 2.82           | 0.07         | 693.1          | <input checked="" type="checkbox"/> | 2.516e1                  | 100.63   |
| 545   | 25          |           | Standard    | <input type="checkbox"/>            | EGC I          | (No IS) |                      |                 | 25.0000              | 4.712e5 | N/A     | N/A        | 9.482e4 | N/A              | 2.80           | 0.07         | 1131.1         | <input checked="" type="checkbox"/> | 2.425e1                  | 97.00    |
| 605   | 50          |           | Standard    | <input type="checkbox"/>            | EGC I          | (No IS) |                      |                 | 50.0000              | 1.092e6 | N/A     | N/A        | 2.555e5 | N/A              | 2.82           | 0.07         | 978.3          | <input checked="" type="checkbox"/> | 5.656e1                  | 113.13   |
| 665   | 50          |           | Standard    | <input type="checkbox"/>            | EGC I          | (No IS) |                      |                 | 50.0000              | 9.034e5 | N/A     | N/A        | 1.801e5 | N/A              | 2.80           | 0.08         | 2287.8         | <input checked="" type="checkbox"/> | 4.676e1                  | 93.52    |
| 725   | 100         |           | Standard    | <input checked="" type="checkbox"/> | EGC I          | (No IS) |                      |                 | 100.0000             | 2.040e6 | N/A     | N/A        | 4.559e5 | N/A              | 2.82           | 0.07         | 3117.3         | <input checked="" type="checkbox"/> | 1.060e2                  | 105.96   |
| 785   | 100         |           | Standard    | <input type="checkbox"/>            | EGC I          | (No IS) |                      |                 | 100.0000             | 1.784e6 | N/A     | N/A        | 3.655e5 | N/A              | 2.80           | 0.07         | 3448.3         | <input checked="" type="checkbox"/> | 9.261e1                  | 92.61    |
| 845   | V_1A        |           | Unknown     | <input type="checkbox"/>            | EGC I          | (No IS) |                      |                 | N/A                  | 9.108e3 | N/A     | N/A        | 1.819e3 | N/A              | 2.47           | 0.08         | 20.2           | <input checked="" type="checkbox"/> | 1.855e-1                 | N/A      |
| 905   | V_1B        |           | Unknown     | <input type="checkbox"/>            | EGC I          | (No IS) |                      |                 | N/A                  | 9.963e3 | N/A     | N/A        | 2.009e3 | N/A              | 2.47           | 0.08         | 26.7           | <input checked="" type="checkbox"/> | 2.300e-1                 | N/A      |
| 965   | V_2A        |           | Unknown     | <input type="checkbox"/>            | EGC I          | (No IS) |                      |                 | N/A                  | 1.220e4 | N/A     | N/A        | 2.721e3 | N/A              | 2.47           | 0.07         | 31.8           | <input checked="" type="checkbox"/> | 3.464e-1                 | N/A      |
| 1025  | V_2B        |           | Unknown     | <input type="checkbox"/>            | EGC I          | (No IS) |                      |                 | N/A                  | 1.267e4 | N/A     | N/A        | 2.789e3 | N/A              | 2.46           | 0.07         | 36.9           | <input checked="" type="checkbox"/> | 3.710e-1                 | N/A      |
| 1085  | V_3A        |           | Unknown     | <input type="checkbox"/>            | EGC I          | (No IS) |                      |                 | N/A                  | 8.442e3 | N/A     | N/A        | 1.508e3 | N/A              | 2.46           | 0.09         | 22.4           | <input checked="" type="checkbox"/> | 1.508e-1                 | N/A      |
| 1145  | V_3B        |           | Unknown     | <input type="checkbox"/>            | EGC I          | (No IS) |                      |                 | N/A                  | 1.153e4 | N/A     | N/A        | 2.284e3 | N/A              | 2.47           | 0.08         | 35.7           | <input checked="" type="checkbox"/> | 3.118e-1                 | N/A      |
| 1205  | V_4A        |           | Unknown     | <input type="checkbox"/>            | EGC I          | (No IS) |                      |                 | N/A                  | 7.841e3 | N/A     | N/A        | 1.677e3 | N/A              | 2.47           | 0.07         | 19.4           | <input checked="" type="checkbox"/> | 1.195e-1                 | N/A      |
| 1265  | V_4B        |           | Unknown     | <input type="checkbox"/>            | EGC I          | (No IS) |                      |                 | N/A                  | 9.377e3 | N/A     | N/A        | 1.594e3 | N/A              | 2.46           | 0.09         | 29.3           | <input checked="" type="checkbox"/> | 1.996e-1                 | N/A      |



Corrente ionica estratta  
dell'Epigallocatechina  
(EGC)

Retta di calibrazione  
Epigallocatechina (EGC)