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USING MICROWAVES AS A PRE-TREATMENT FOR ENHANCING THE EXTRACTION OF POLYPHENOLS FROM GRAPE STEMS

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Abstract.
Grape stems are a residual woody material from the vinification process. A waste that, if it is not treated in a correctly manner, could represent an environmental problem. Moreover, in the last years, special attention has been paid to this waste due to its high content of polyphenols, mainly stilbenes and flavonoids. Several studies have already revealed the potential and possibilities of these compounds in industries, such as alimentary, cosmetic and pharmaceutic thanks to their antioxidant, antimicrobial and/or anticarcinogenic properties. In this work, the effect of solid-liquid extraction parameters of polyphenols from grape stems have been studied. These parameters were: solid-liquid ratio (R_{S-L}), type of solvent (variation of the percentage of ethanol in the hydroalcoholic mixture) and temperature. Parameter values selected as the best for polyphenol extraction in a conventional solid-liquid extraction were: a R_{S-L} of 0.10 g/mL, a temperature of 75ºC and a hydroalcoholic mixture with a 50% vol. of ethanol. Furthermore, microwaves were applied to grape stems as a pre-treatment prior to the conventional extraction for the first time. In this case the parameters assessed were also solid-liquid ratio, type of solvent and, in addition, the time of the pre-treatment. For this purpose, a statistical surface design was employed to obtain the optimum conditions which maximize the final TPC of the extracts. In a first approach, extracts were characterized in terms of total polyphenol content and total flavonoid content. The main result was that, microwaves make it faster the extraction (until 4 times) but it is not clear their role on the extraction yield.

Key-words. Grape stems, stilbenes, flavonoids, microwave pre-treatment, process intensification

INTRODUCTION

Among the diverse by-products from the winemaking process, the woody vine material represents around a 12% of the 2-3 million tons of wastes generated per year only in Spain [1]. Normally, these residues are composted or burned for disposal, constituting an environmental problem [2]. Nevertheless, they represent an important source of phenolic compounds, mainly stilbenes and flavonoids. Both families of phenolic compounds have a high reputation among those phytochemicals with health-promoting effects such as, antioxidant, anticarcinogenic, cardioprotective and antimicrobial [3]. Thus, these polyphenols present a potential use as food constituents, antioxidants, cosmetics or drug adjuvants. In the last years, there have been a lot of studies focused on highlighting the power of grape canes as an alternative and natural source of polyphenols, contributing to the development of new wine-related products and could also lead to a sustainable growth of the wine industry. Several authors have investigated the extraction of stilbenes and other phenolic compounds from grape stems. As it is worldwide known, solid-liquid extraction is the most implemented process to extract bioactive compounds from a vegetable matrix. For the case of grape stems, this procedure has been used in many studies with different types of organic solvents such as dichloromethane, methanol, ethanol and/or water and their mixtures [2], [4]. Many times, the solvent extraction has been combined with the application of vortex and sonication in order to enhance the polyphenol extraction [5]. As substances of interest have generally an intracellular localisation, the enhancement of the internal mass transfer will improve the extraction of the substances. This boost of the internal mass transfer can be achieved thanks to the employment of cell disruption methods like ultrasounds or microwaves. For the case of grape stems, ultrasound-assisted extraction have been performed [6], [7] as well as microwave (MW) assisted extractions [3] for the recovery of polyphenols. Both extraction methods showed increments on the recovery of polyphenols compared with those obtained using a solid−liquid method. As an alternative technique, some authors have suggested using

MW pre-treatment to the conventional extraction of polyphenols in which low residence time pre-treatments (below 120s) provide a better homogeneity of the energy absorb by the material [8].
From the best of our knowledge, MW have never been applied as a pre-treatment to grape stems. So, this work is aimed at the recovery of polyphenols from grape stems using MW as a pre-treatment prior to the conventional extraction. Firstly, the main parameters that govern a solid-liquid extraction are studied, taking into account the results from the literature. Then, MW pre-treatments are presented, varying the main parameters using a statistical surface design (Box-Benkhen) to obtain the optimum conditions which maximize the final total phenolic content (TPC) of the extracts. Evaluation of the extraction yields are given in term of TPC and in total flavonoid content (TFC).

MATERIALS AND METHODS

Materials

Raw material: Grape stems were kindly provided by Matarromera winery (Valladolid, Spain) in November 2017, after the grape harvest. Stems were dry in an oven at the temperature of 65ºC for 24 hours. Dry stems were ground using a chopper (A320R1, Moulinex) and two different fractions were obtained. On the one hand, small pieces with an average length of 6mm were collected. On the other hand, a fine powder (67.4µm) was achieved. Both fractions were stored at room temperature, protected from light. **Solvents**: used for extractions were absolute ethanol (99.9% Carlo Erba Reagents, Val de Reuil, France), bidistilled water (Milli-Q® Integral) and hydrochloric acid (≥37%, puriss. p.a., Riedel-de Haën, France)

Methods

Conventional solid-liquid (S-L) extractions were performed varying the parameters: solid-liquid ratio (0.10, 0.07, 0.04 g/mL), type of solvent (ethanol and hydroalcoholic mixtures varying the % of ethanol in 80, 50 and 20%) and temperature (25, 50 and 75ºC). Solvent volume was fixed in 75 mL and the stems mass was varied according to the different $R_{S\text{-}L}$ (7.5, 5.25, 3 g, respectively) All the S-L extractions were performed with an agitation of 300 rpm for 60 minutes. pH was adjusted to 2.5 with HCl. Furthermore, particle size was also studied as both fractions (pieces and powder) of ground stems were used for the solid-liquid extractions.
MW pre-treatments were carried out in a CEM Discovery Microwave with a fixed power of 300W. A statistical surface response design was performed using Statgraphics® Centurion XVII software in order to obtain the optimum conditions which maximize the final TPC and TFC of the extracts. Studied parameters were: solid-liquid ratio (0.25, 0.50 and 0.75 g/mL), solvent mixture (hydroalcoholic mixtures varying the percentage of ethanol and time of microwaves applied (30, 60 and 90s). After the pre-treatment, a conventional solid-liquid extraction is performed at best extraction conditions.
Extracts characterization. TPC was performed by Folin-Ciocalteou [9] method and expressed as milligrams of gallic acid equivalents per grams of dry stems. TFC was evaluated using also a colorimetric method [10]. Both TPC and TFC were measured along time in order to build extraction kinetics curves.

RESULTS AND DISCUSSON

Conventional solid-liquid extractions

First of all, the $R_{S\text{-}L}$ was studied for both stems fractions, pieces and powder. TPC and TFC were measured for each solid-liquid extractions performed at 25ºC and with a hydro-alcoholic mixture of 80% EtOH (%vol.) and different $R_{S\text{-}L}$. Samples were gathered along 60 min to build extraction kinetics curves. Figure 1 shows TFC values along time for the different $R_{S\text{-}L}$.

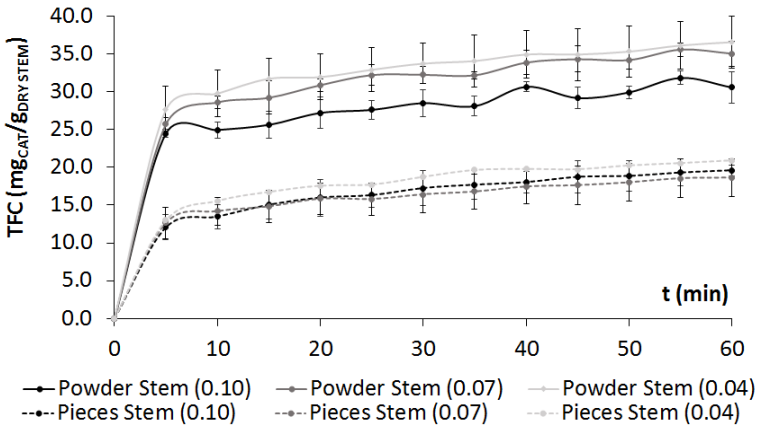

Figure 1: kinetic extraction curves of TFC values for the studied $R_{S\text{-}L}$ (0.10, 0.07 and 0.04) after 60 minutes of extraction with stem powder and stem pieces at 25ºC and with a hydro-alcoholic mixture of 80% EtOH (%vol.).

As it was expected, TFC values are higher for stem powder than when stem pieces were used. This discrepancy is assumed to be a consequence of a diffusional stage in the bigger particles. Thus, the rest of the parameters studied in the solid-liquid extractions, were carried out with stem powder. Table 1, shows TPC and TFC values for the different variables. Finally, parameters selected as the best for polyphenols extraction from stem powder were a R_{S-L} of 0.10 g/mL, a hydroalcoholic mixture with a 50% vol. of ethanol and a temperature of 75ºC.

Table 1: TPC and TFC values for variations of each parameters after 60 minutes of extraction with stem powder. Values in italics represent the highest achieved concentrations.

		TPC ($mg_{GAE}/g_{DRY\ STEM}$)	TFC ($mg_{CAT}/g_{DRY\ STEM}$)
R_{S-L} (g/mL)	**0.1**	34.6 ± 0.5	30.6 ± 2.1
	0.07	37.1 ± 0.4	35.0 ± 1.7
	0.05	36.2 ± 0.0	36.6 ± 3.4
Solvent type (% vol. EtOH)	**80**	34.6 ± 0.5	30.6 ± 2.1
	50	49.0 ± 2.0	46.0 ± 0.7
	20	40.1 ± 0.7	36.4 ± 1.3
Temperature (ºC)	**25**	49.0 ± 2.0	46.0 ± 0.7
	50	57.5 ± 1.5	55.3 ± 2.3
	75	*65.5 ± 0.5*	*63.8 ± 4.7*

Microwave pre-treatments extractions

MW pre-treatments were firstly performed for stem powder as the solid-liquid extraction variables have been already studied. Preliminary MW experiments for stem powder did not show an enhancement on the TPC and TFC yields the final extracts. Nevertheless, a decrease in the required time to achieve a certain concentration is achieved when MW are applied (up to 4 times lower). This effect can be seen in Figure 2 since MW accelerates the extraction of flavonoids. The result was that a no clear enhancement on the extraction yield was be achieved. This fact can be due to the small particle size and high concentration of the polyphenols in the raw material decreasing the internal mass transfer limitation. In contrast, MWs are expected to have a mandatory role with the stem pieces because of their higher size, which also are the major fraction obtained from the milling. Furthermore, these stem pieces would also be the most attractive way from an industrial point of view.

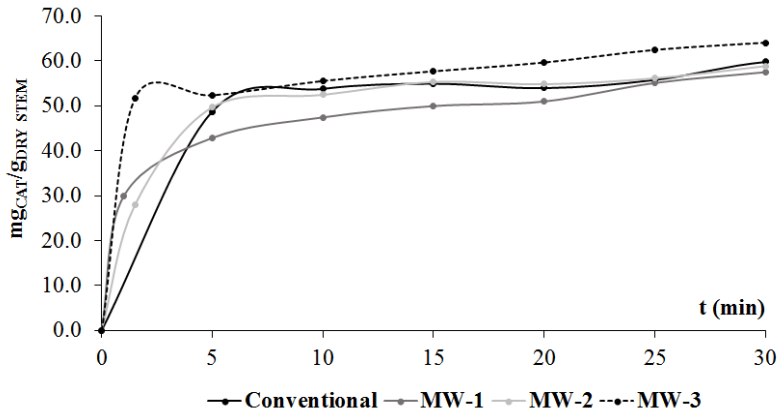

Figure 2: comparison of the kinetic extraction curves of TFC values of the conventional extraction (50:50, EtOH:H_2O; 75ºC ; 0.10 g/mL) and MW pre-treatment experiments MW-1(30s, 80:20, EtOH:H_2O; 0.50 g/mL), MW-2(90s, 50:50, EtOH:H_2O; 0.50 g/mL), MW-3 (90s, 50:50, EtOH:H_2O; 0.10 g/mL).

CONCLUSIONS

A whole study about the polyphenol extraction from grape stems has been performed. The best yield (TPC: 65.5 ± 0.5 $mg_{GAE}/g_{DRY\ STEM}$; TFC: 63.8 ± 4.7 $mg_{CAT}/g_{DRY\ STEM}$) was obtained at the following conditions: R_{S-L} of 0.10 g/mL, a hydroalcoholic mixture with a 50% vol. of ethanol and a temperature of 75ºC. MWs accelerated the extraction kinetics (up to 4 times) but had a minor effect on polyphenol extraction yield (%) was achieved for the powdery raw material due to the absence of internal mass transfer limitation. The effect of MWs on stem pieces is expected to be higher.

ACKNOWLEDGEMENTS

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Universidad de Valladolid - Spain

Outline

Introduction	Winery residues, types and quantities
Objectives	Main goal of this research
Materials and Methods	Grape stems, procedures
Results	Main yields and results
Conclusions	General conclusions

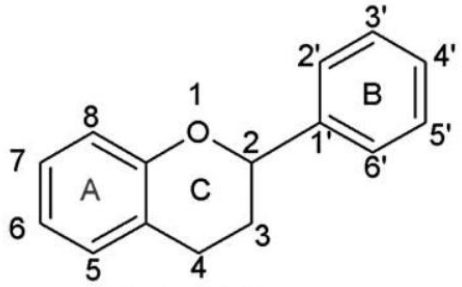
Winery residues

$3.5 \cdot 10^5$ tons of grape stems generated per year only in Spain

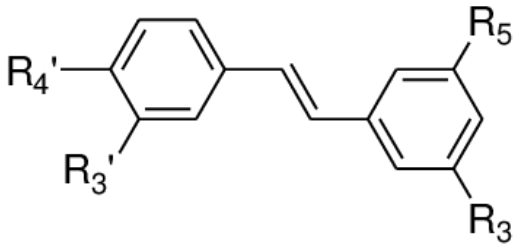
composted or burned for disposal → environmental problem

Phenolic compounds

Flavonoids



Stilbenes



Health-promoting effects

- Antioxidant
- Anti-carcinogenic
- Anti-microbial
- Anti-fungical
- Cardio-protective

food constituents, cosmetics or drug adjuvants

sustainable growth of the wine industry

Objectives

1. Polyphenol extraction maximization :

- Total polyphenol and flavonoid extraction kinetics
  - Solid-liquid ratio, R_{S-L} (g/mL)
  - Solvent composition (% ethanol hydroalcoholic mixtures)
  - Temperature (ºC)
- Microwave (MW) pre-treatments

Statistical surface area response
  - Solid-liquid ratio, R_{S-L} (g/mL)
  - Solvent composition (% ethanol hydroalcoholic mixtures)
  - Time (s)

2. Identification of main compounds present in grape stems

Grape stems

- Grape Stems (GS) → dry at 65ºC for 24 hours.
- GS were ground using a chopper.
- Small pieces with an average length of 3-6mm

Extract characterization

- **Total Polyphenol Content - TPC**
  - Folin-Ciocalteou ($mg_{GAE}/g_{DRY\ STEM}$)
- **Total Flavonoid Content - TFC**
  - Aluminium Complexation Reaction ($mg_{CATE}/g_{DRY\ STEM}$)
- **ORAC**
  - µmol of Trolx Equivalents per gram of dry extract ($\mu mol_{TE}/g_{DRY\ STEM}$)
- **HPLC-DAD and MS/MS-MRM**
  - Identification of main compounds

iBET Instituto de Biologia Experimental e Tecnológica

Extraction kinetics

S-L extraction

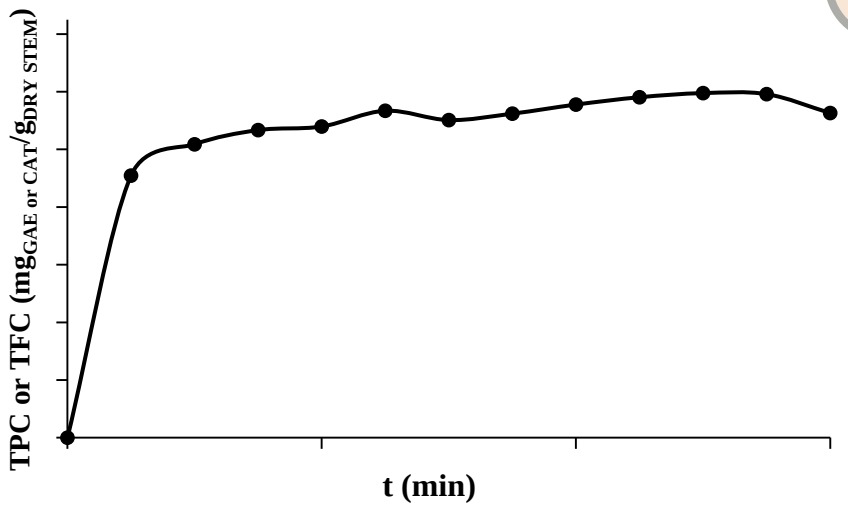
- Conventional **polyphenol** extraction kinetics curve.

Fixed Conditions

- 300 rpm
- pH = 3.0
- t = 60 min

Parameters

- Solid-liquid ratio (g/mL): 0.07, 0.04, 0.02
- Solvent (%EtOH): hydro-alcoholic mixtures (20, 50, 80)
- Temperature: 25, 35 and 45ºC.



- TPC - Folin-Ciocalteou
- TFC - spectrophotometric method

MW pre-treatment

Total of 15 experiments
- three levels (-1, 0, 1)
- triplicate of the central point

Three main variables:
- Solid-liquid ratio (g/mL): 0.10, 0.30, 0.50
- Solvent composition (% EtOH): 20, 50, 80
- Time of microwaves (s): 30, 60, 90

Pre-treatment

- Statistical surface area response (Box-Behnken)

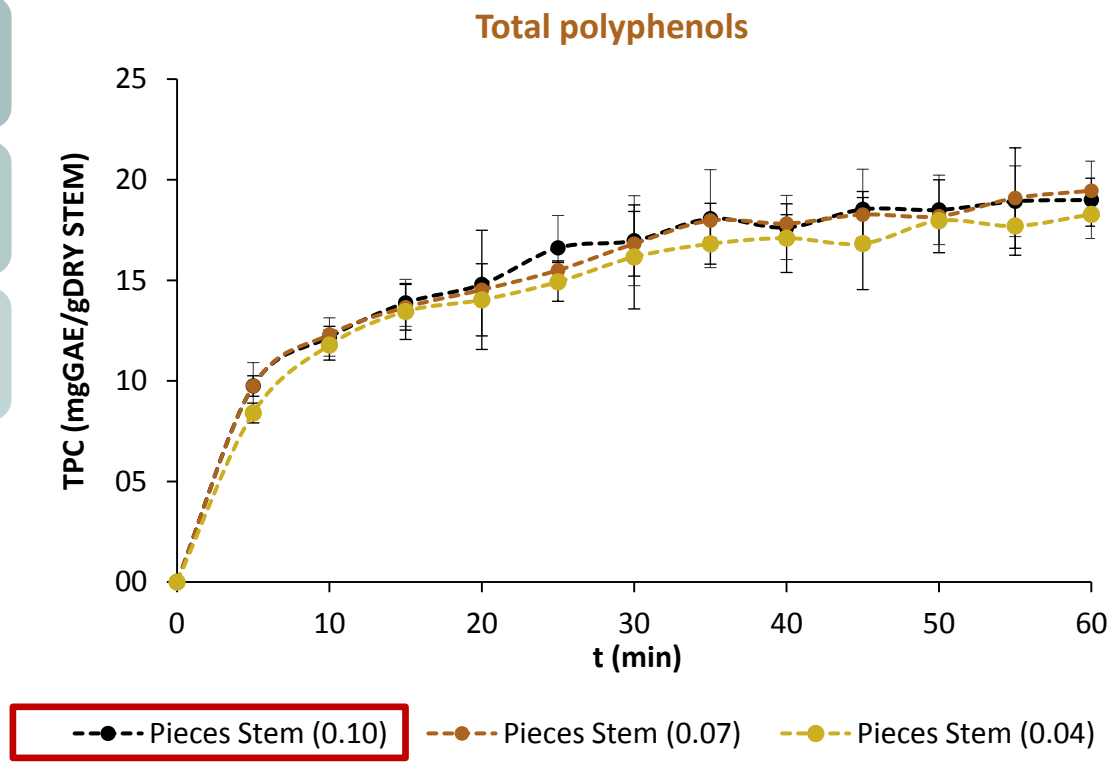
Data

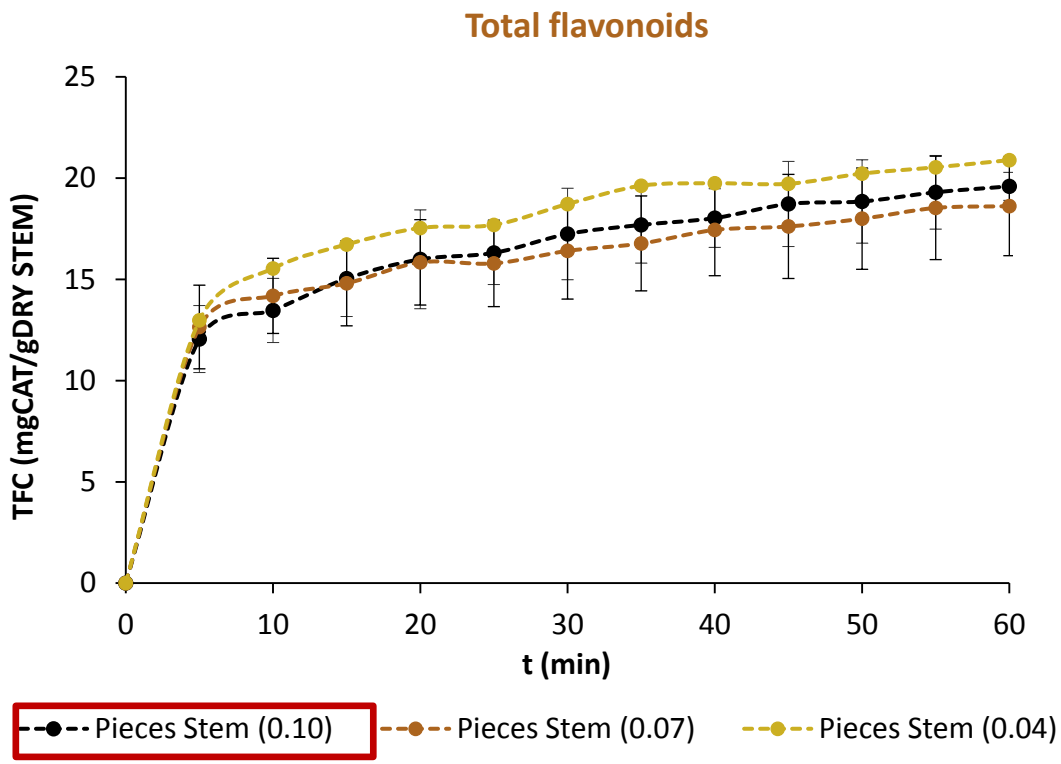
$$Y = \beta_0 + \sum_{j=1}^{k} \beta_j X_j + \sum_{j=1}^{k} \beta_{jj} X_j^2 + \sum_{i=1}^{k} \sum_{j=1}^{k} \beta_{ij} X_i X_j$$

MW optimum point

Kinetic extraction curves

Study of the solid-liquid ratio (at 25ºC, 20:80 (%vol.) H_2O:EtOH)

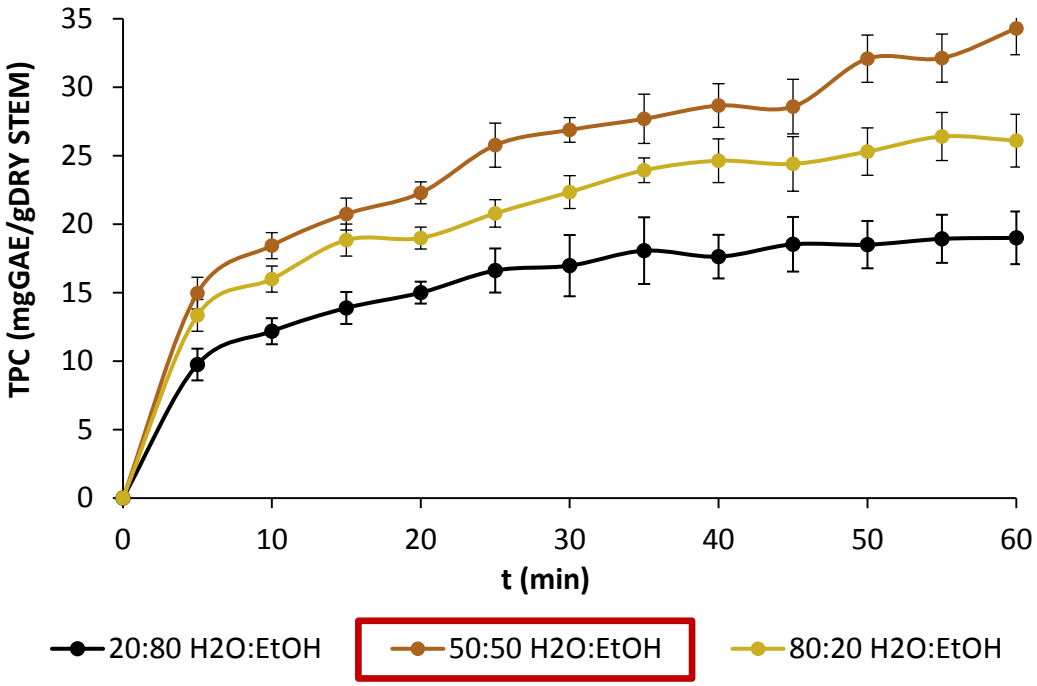




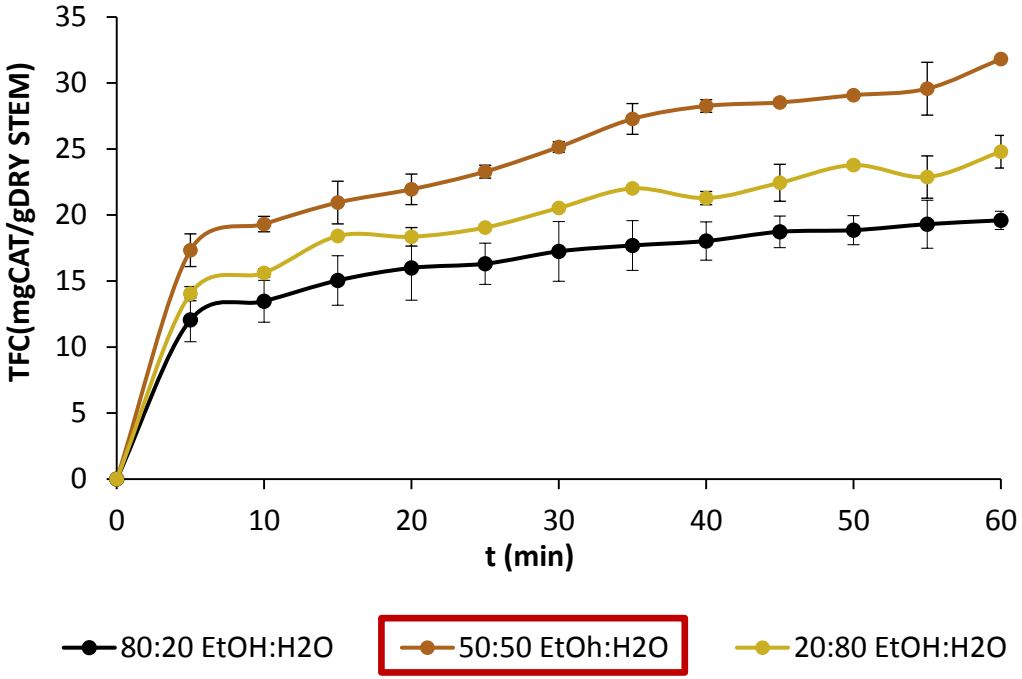
Kinetic extraction curves

Study of the solvent composition (at 25º, R_{S-L} = 0.10 g/mL)

Total polyphenols

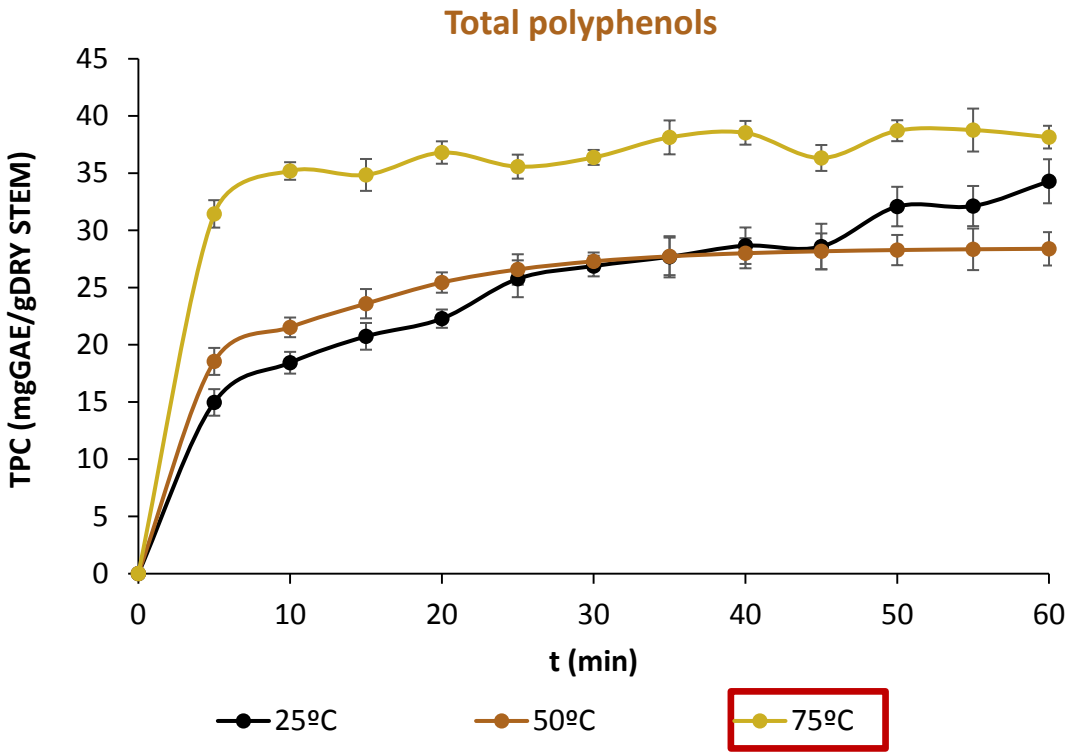


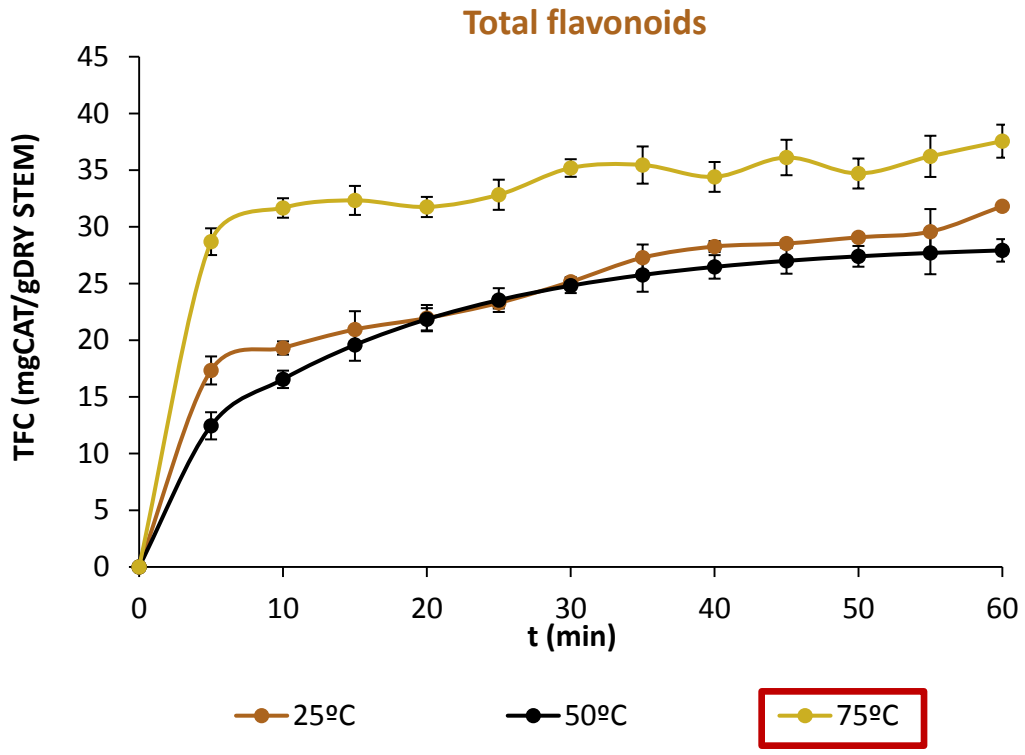
Total flavonoids



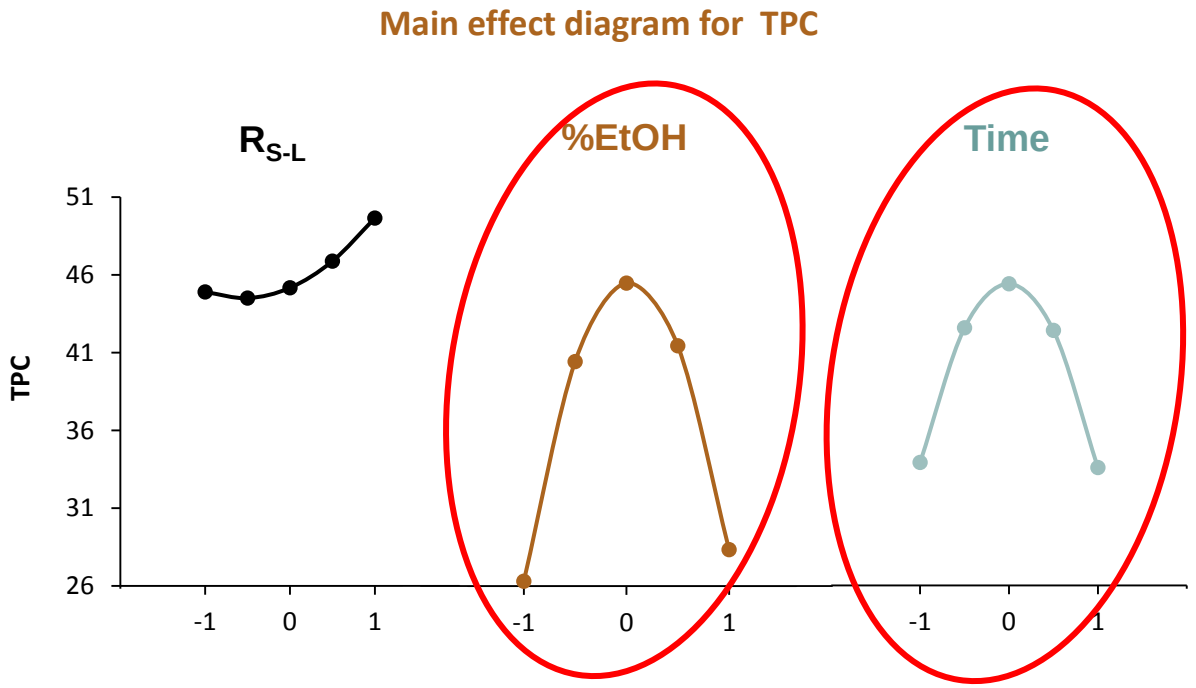
Kinetic extraction curves

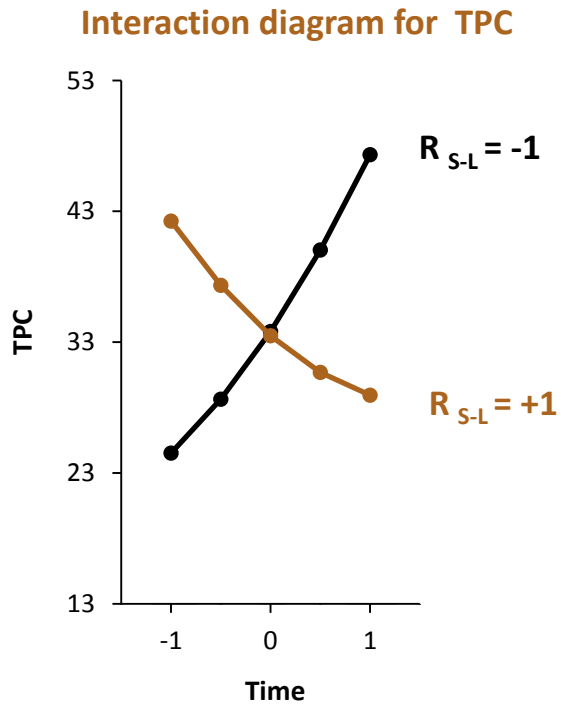
Study of the temperature (R_{S-L} = 0.10 g/mL and 50:50 (%vol.) EtOH:H2O)





MW pre-treatments





	-1	0	1
R S-L	0.1	0.3	0.5
%EtOH	20	50	80
Time	30	60	90

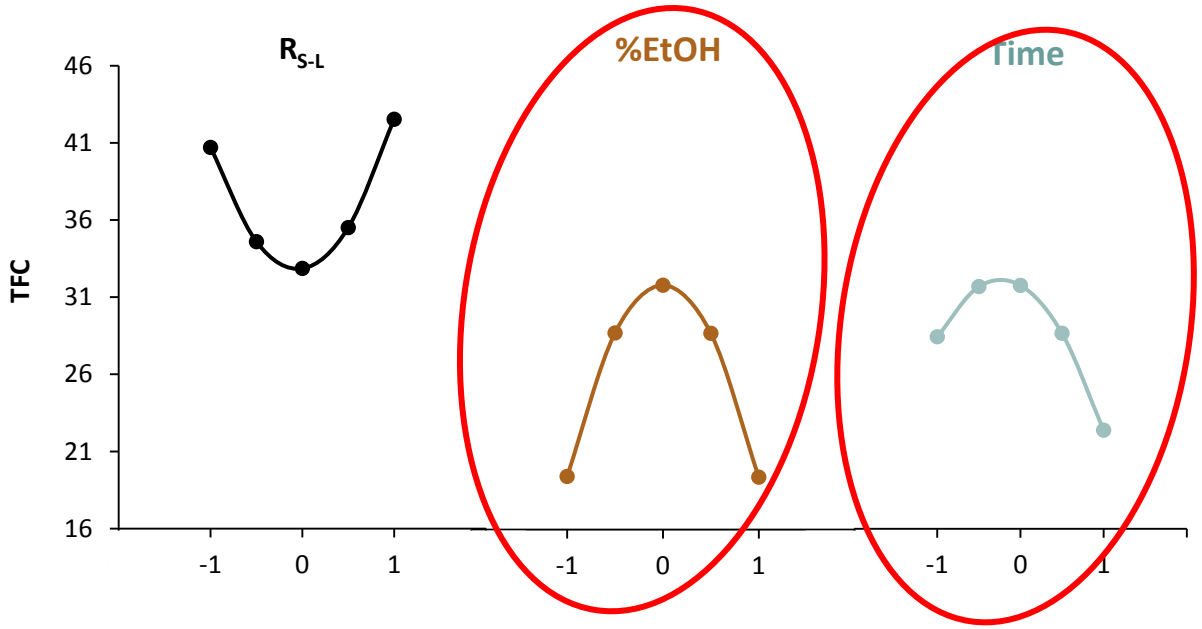
$R^2 = 84\ \%$

Optimum point for TPC

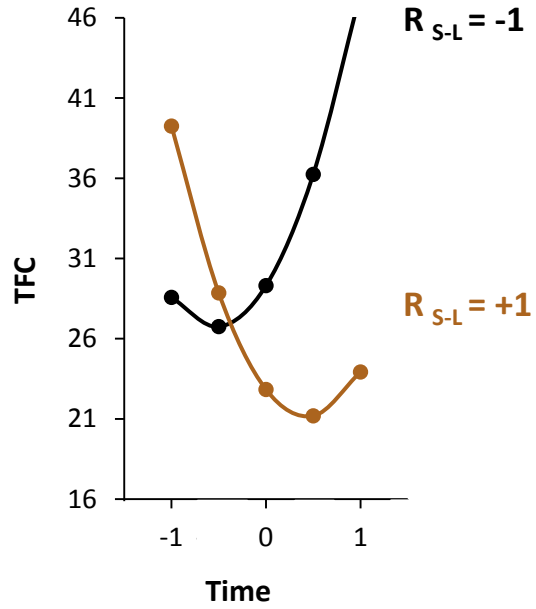
- Solid-liquid ratio (g/mL): 0.50
- Solvent composition (% EtOH): 59.944
- Time of microwaves (s): 35.79

MW pre-treatments

Main effect diagram for TFC



Interaction effect diagram for TFC



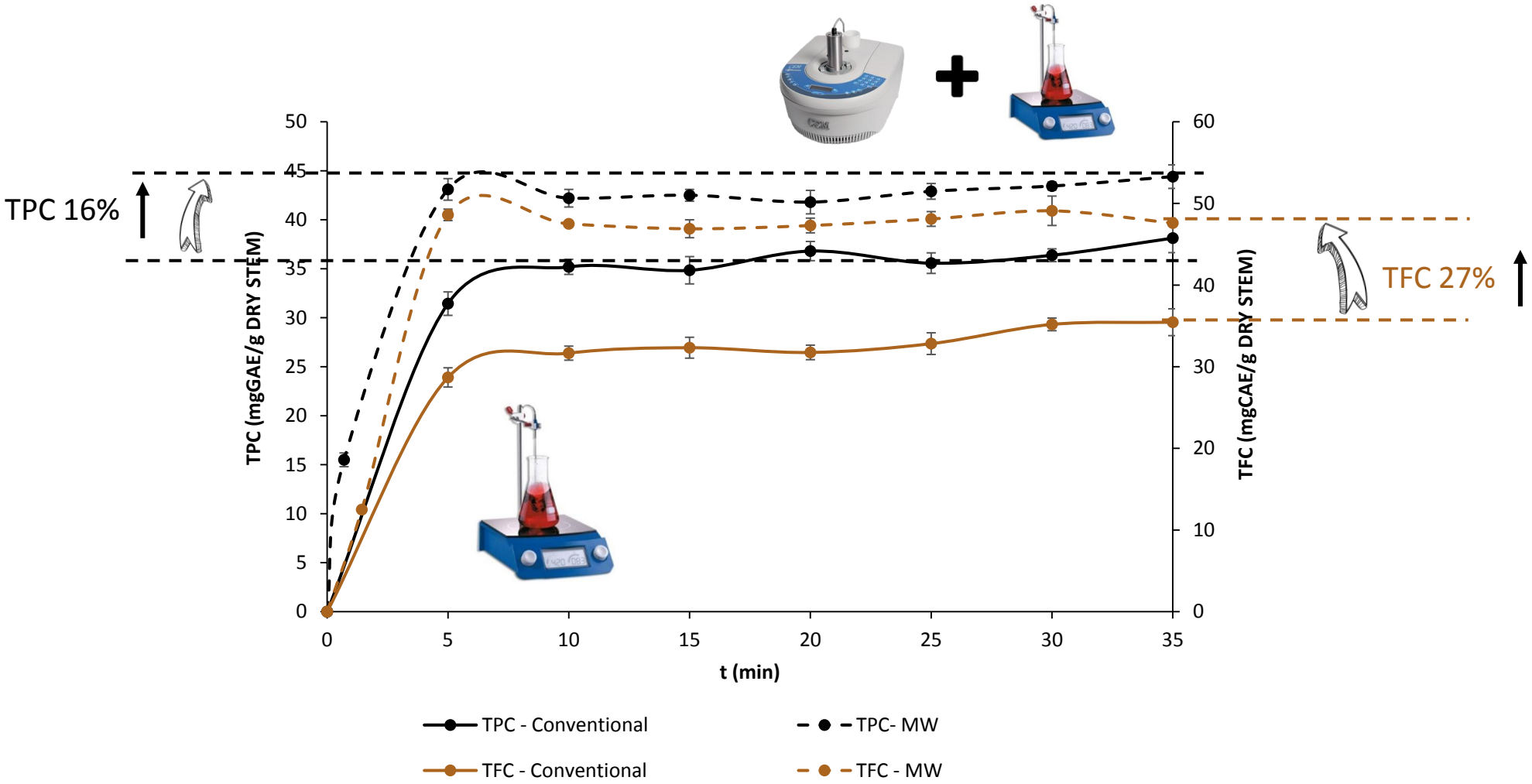
	-1	0	1
R_{S-L}	0.1	0.3	0.5
%EtOH	20	50	80
Time	30	60	90

$R^2 = 87\ \%$

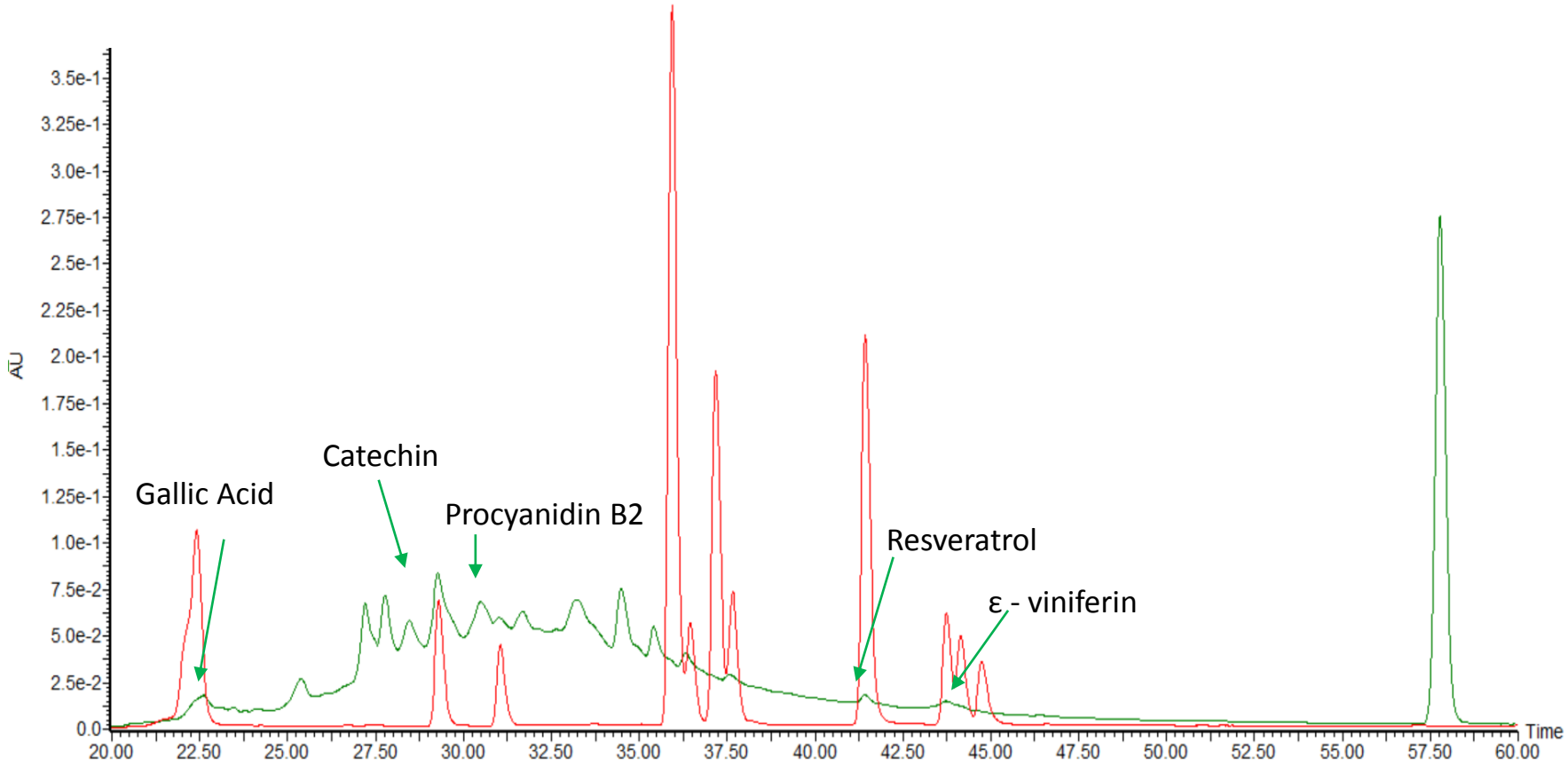
Optimum point for TFC

- Solid-liquid ratio (g/mL): 0.10
- Solvent composition (% EtOH): 57.03
- Time of microwaves (s): 85.71

MW pre-treatments



HPLC



ORAC

	ORAC µmol $_{TE}$/g $_{DRY\ STEM}$
Conventional	791 ± 90
MW - TPC optimum	848 ± 110
MW - TFC optimum	1056 ± 56

Conclusions

- **Grape stems are a suitable source of polyphenols → 38.2 ± 1.0 $mg_{GAE}/g_{DRY\ STEM}$**
  flavonoids → 37.6 ± 1.5 $mg_{CATE}/g_{DRY\ STEM}$

- **Solid-liquid extraction best conditions:**
  - R_{S-L}(g/mL): **0.10**
  - Solvent: **50:50 EtOH:H2O (% vol.)**
  - T^a: **75ºC**
  - Time: **60 min**

- **Use of MW as pre-treatments → enhance TPC and TFC extraction**

→ MW increases TPC in a **16%.**

→ MW increases TFC in a **27%.**

→ Reduces operational times from ~ 60 min to **5 min.**

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USING MICROWAVES AS A PRE-TREATMENT FOR ENHANCING THE EXTRACTION OF POLYPHENOLS FROM GRAPE STEMS

Thanks for your attention