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Article

Kinetic Modelling for Flavonoid Recovery from Red Grape (*Vitis vinifera*) Pomace with Aqueous Lactic Acid

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Abstract: This study was undertaken with the aim of establishing a correlation between the extraction yield in total flavonoids from red grape pomace and the extraction temperature, using 0.5% (*w*/*v*) aqueous lactic acid as the solvent system. Extraction of flavonoids was found to obey second-order kinetics, and on such a basis, the yield in total flavonoids at saturation could be very effectively determined and correlated with temperature using non-linear regression. The results indicated that the extraction yield at saturation is highly correlated with temperature, following a quadratic function. The extract obtained at 40 °C had an optimal predicted total flavonoid yield of 13.27 mg rutin equivalents per gram of dry weight, and it was further analyzed by liquid chromatography-mass spectrometry to characterize its major constituents. The polyphenols detected were flavanols, flavonols and an anthocyanin. The outcome of this study outlined that temperatures above 40 °C are rather unfavorable for flavonoid extraction from red grape pomace, as suggested by the model established through kinetics.

Keywords: extraction kinetics; flavonoids; liquid chromatography-mass spectrometry; red grape pomace

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1. Introduction

Winemaking generates a very large amount of residues, characterized by a high content of biodegradable compounds. The residues consist of the remains of de-stemmed grapes, the sediments obtained during clarification, bagasse from pressing and lees, which are obtained after different decanting steps. These wastes may have phytotoxic effects if applied to crops, and thus, pollution problems associated with winery waste treatment and disposal have raised serious concerns about subsurface flow and the impact on the surrounding ecosystems [1].

This waste material has been extensively investigated over the last few years, given its exceptionally high levels for a spectrum of polyphenolic phytochemicals, which possess bioactivities beneficial to human health; yet, the methodologies for efficient, but also environmentally compatible, recovery of these substances may suffer serious shortcomings, such as the toxicity of the solvents used (methanol, acetone), the need for solvent removal and recycling (ethanol) and cost (supercritical fluids). However, the search for plant food residues as cost-effective sources of multifunctional phytochemicals embraces valorization strategies in the direction of: (i) exploiting materials with a high content and a rich composition; (ii) deploying effective recovery processes; and (iii) ascertaining the production of novel formulations without further generation of waste, such as waste solvents, which pose severe environmental risks [2]. In this line, the use of low-cost, non-toxic solvent systems for the recovery of target compounds becomes imminent [3,4].

Organic acids, such as citric acid, are non-toxic natural food constituents and, owing to their relatively low pK_a, they may serve as very effective pH regulators. This has been a significant factor in the use of environmentally-benign solvents, employed for the extraction of bioactive polyphenols from various sources, because it has been observed that pH modification might affect extraction selectivity [5–8]. In particular, it has been demonstrated that basic pH in low-ethanol media was more favorable for polyphenol extraction from red grape pomace (RGP) [9]. Furthermore, studies on the extraction of polyphenols from grape stems using water/ethanol solutions showed that efficient flavanol extraction required a lower pH, as opposed to total flavonoid, whose extraction was facilitated at a pH higher than 4.5 [10]. The mechanism by which pH may modify polyphenol extraction selectivity is not clear, but earlier reports suggested that in a moderately acidic environment (pH > 5), the most acidic phenolic hydroxyls on the polyphenol skeleton might be weakly dissociated, thus becoming more soluble [6]. This could enable more effective transfer of polyphenols from the solid particles into the liquid phase, hence the increased yields observed.

The present study was undertaken to investigate the potential of aqueous solutions of lactic acid to act as an efficient, non-toxic means of extracting polyphenols from red grape pomace. The experimental setup was based on the optimization of acid concentration and resident time, taking into consideration the total flavonoid yield of the extracts obtained. Kinetics and non-linear regression analysis were employed to define a set of conditions that may be used for further engineering the extraction process. Some principal polyphenols detected in the richest extract obtained were tentatively identified using liquid chromatography-mass spectrometry.

2. Experimental Procedure

2.1. Chemicals

All solvents used for chromatographic purposes were HPLC grade. Rutin (quercetin 3-*O*-rutinoside) was from Sigma Chemical Co. (St. Louis, MO, USA). Lactic acid (LA) and aluminum chloride ($AlCl_3$) were from Merck (Darmstad, Germany).

2.2. Red Grape Pomace

Grape pomace originating from vinification of the Agiorgitiko variety (*Vitis vinifera* spp.) was kindly provided by the Department of Food Science and Human Nutrition, Agricultural University of Athens. The pomace was dried in an oven at 65 °C for 48 h and then pulverized into a fine powder (approximate mean particle diameter: 0.3 mm) in a laboratory mill. Following drying, the moisture content of RGP was estimated to be 55% ± 2%. The pulverized material was kept at −20 °C until use.

2.3. Extraction Procedure

An aliquot of 2.4 g of pulverized RGP was added in 120 mL of 0.5% (*w*/*v*) LA solution, in a 250-mL glass vial, and extractions were performed under stirring with a Teflon-coated magnetic stirrer, at 80 rpm and various temperatures (30, 40, 60 °C). Sampling was carried out by removing 1 mL of extract at predetermined intervals over a period of 320 min, according to the experimental design. The concentration of LA solution used, as well as the range of the extraction period, was based on preliminary experimentation.

2.4. Total Flavonoid Yield (Y_{TFn})

A previously reported protocol was used [10], with modifications. An aliquot of 0.05 mL $AlCl_3$ (2% in 5% acetic acid in methanol) was mixed with 0.5 mL sample and 0.5 mL 5% acetic acid in methanol. The mixture was left for 30 min at room temperature, and the absorbance was measured at 415 nm. Determination of the total flavonoid concentration (C_{TFn}) in the extracts obtained, expressed in $mg \cdot L^{-1}$, was carried out using rutin (quercetin 3-*O*-rutinoside) as the calibrating standard. The total flavonoid yield, Y_{TFn}, was determined as mg rutin equivalents (*RtE*) per g dry pomace weight (*dpw*), as follows:

$$Y_{TFn}\ (mg\ RtE\ g^{-1} dpw) = \frac{C_{TFn} \times V}{m} \quad (1)$$

where V is the volume of the extract (in mL) and m the dry pomace weight (in *g*).

2.5. Kinetics and Statistical Analyses

All determinations were carried out at least in triplicate, and values were averaged and given along with the standard deviation (±SD). Kinetics was performed by carrying out non-linear regression between Y_{TFn} and t values. Kinetics and linear and non-linear correlations were established at least at a 95% significance level ($p < 0.05$). For all statistics, SigmaPlot™ (London, UK) 12.0 and Microsoft Excel™ (Redmond, WA, USA) 2010 were used.

3. Results and Discussion

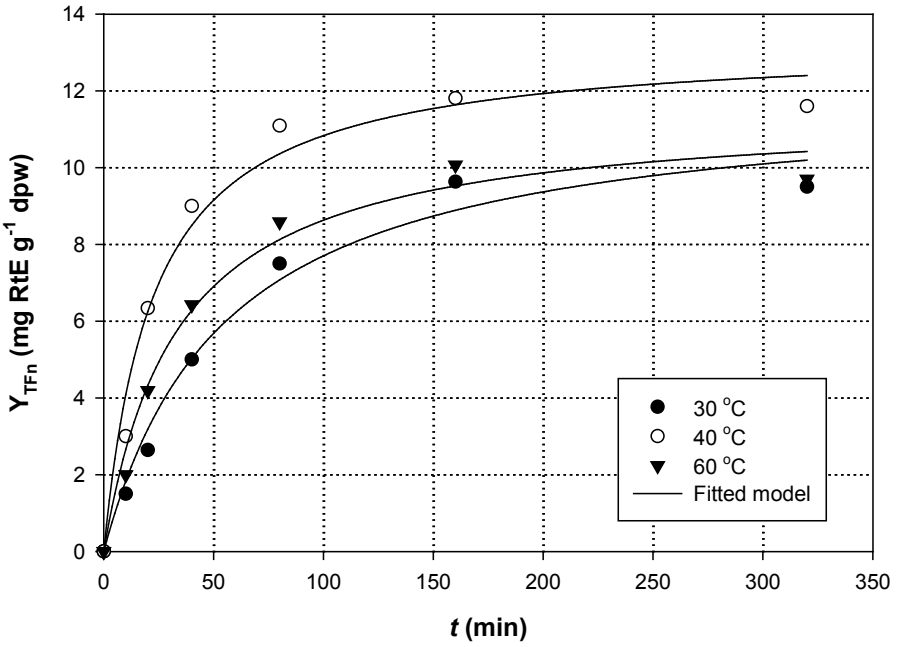
3.1. Kinetics of TFn Extraction

The curve fitted to the experimental data (Figure 1) described the second-order extraction kinetics, considering the boundary conditions $t = 0$ to t and $Y_{TFn(t)} = 0$ to $Y_{TFn(t)}$ [3]:

$$Y_{TFn(t)} = \frac{Y_{TFn(s)}^2 kt}{1 + Y_{TFn(s)} kt} \quad (2)$$

where $Y_{TFn(s)}$ and k represent the *TFn* yield at saturation and the extraction rate constant, respectively. Fitting for every temperature tested was statistically significant ($R^2 > 0.97$, $p < 0.0001$), suggesting that Equation (2) can adequately predict Y_{TFn} as a function of t.

Figure 1. Time course of total flavonoid yield (Y_{TFn}) during the extraction of red grape pomace (RGP) using 0.5% (*w*/*v*) aqueous lactic acid (LA) at various temperatures. Extractions were carried out at 80 rpm and a liquid-to-solid ratio of 50 mL·g^{-1}.



By admitting the assumptions that (i) polyphenols leached from the solid particles into the solution through diffusion and (ii) at saturation conditions, Y_{TFn} remained constant, the second-order extraction kinetics is rather indicative of two extractions phases: first, diffusion was fast, most probably due to the fast solubilization of polar phenolics and the high concentration gradient, as stated by Fick's law; second, diffusion was slowed down, because of the lower concentration gradient and the longer time required for the extraction of the less polar or the less easily-extracted compounds.

Transformation of Equation (2) provides a linearized form:

$$\frac{t}{Y_{TFn(t)}} = \frac{1}{kY_{TFn(s)}^2} + \frac{t}{Y_{TFn(s)}} \quad (3)$$

When t approaches 0, the initial extraction rate, h, given as $Y_{TFn(t)}/t$, is defined as:

$$h = kY_{TFn(s)}^2 \quad (4)$$

Plotting $t/Y_{TFn(t)}$ *versus* t would give a straight line in the form of $y = ax + b$ (Figure 2), where $a = 1/Y_{TFn(s)}$ and $b = 1/h$. Thus for each case examined, $Y_{TFn(s)}$, k and h could be determined graphically. For all temperatures tested, the correlations between $t/Y_{TFn(t)}$ and t were very high and statistically significant ($R^2 > 0.99$, $p < 0.0001$), which permitted the reliable determination of the kinetic parameters (Table 1).

Figure 2. Second-order kinetics of *TFn* extraction from RGP using 0.5% (*w*/*v*) aqueous LA. Extractions were carried out at 80 rpm and a liquid-to-solid ratio of 50 mL·g^{-1}.

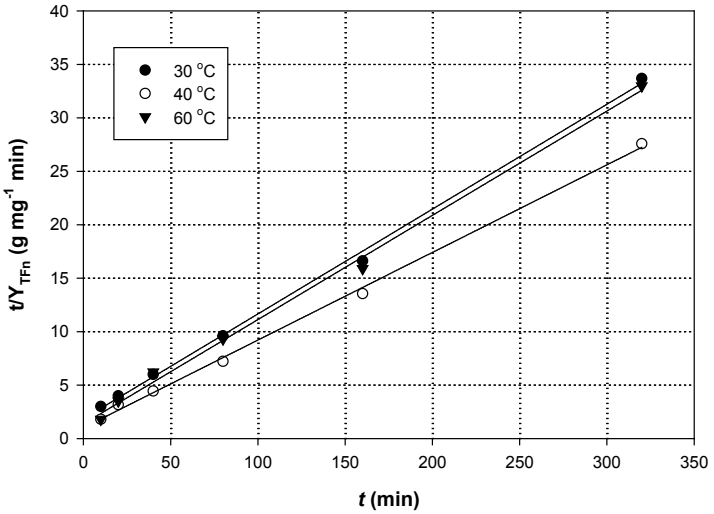


Table 1. Parameters of second-order kinetics, determined for the Y_{TFn} using 0.5% (*w*/*v*) aqueous lactic acid, at the temperatures tested. dpw, dry pomace weight; RtE, rutin equivalents.

T (°C)	Kinetic Parameters		
	k (g·mg^{-1}·min^{-1}) × 10^{-3}	h (mg·g^{-1}·min^{-1})	$Y_{TFn(s)}$ (mg·RtE·g^{-1}·dpw)
30	1.51	0.22	11.98
40	3.35	0.59	13.27
60	2.62	0.35	11.50

The overall extraction constant k showed an increasing tendency from 30 to 40 °C, but declined by shifting the extraction temperature from 40 to 60 °C. This phenomenon was consistent for h and $Y_{TFn(s)}$, indicating that temperatures above 40 °C did not favor flavonoid extraction, resulting in poorer Y_{TFn} due to a reduced extraction rate. Previous studies reported similar results regarding anthocyanin extraction, where temperatures above 35 °C gave reduced extraction yields [11]. Likewise, total polyphenol extraction from onion solid wastes was negatively affected by temperatures higher than 40 °C [12]. However, contradictory results have also been reported [13].

In order to better illustrate the effect of both t and T on Y_{TFn}, the building of a kinetic model was attempted. Non-linear regression between $Y_{TFn(s)}$ and T values was shown to obey a quadratic function. This function was described by the following equation:

$$Y_{TFn(s)} = -0.59 + 0.64T - 0.0072T^2 \ (R^2 = 1.000, p < 0.0001) \quad (5)$$

Thus, using Equation (5), $Y_{TFn(s)}$ can be very reliably predicted as a function of T, within the range 30–60 °C. In the same fashion, non-linear regression between h and T values was shown to obey a quadratic correlation, described by the following equation:

$$h = -0.76 + 0.064T - 0.0008T^2 \quad (6)$$

After rearrangement of Equation (3), Y_{TFn} at any time, t, can be calculated:

$$Y_{TFn(t)} = \frac{t}{\frac{1}{h} + \frac{t}{Y_{TFn(s)}}} \quad (7)$$

Combining Equations (5)–(7), the following mathematical model is obtained:

$$Y_{TFn(t,T)} = \frac{t}{\frac{1}{-0.0008T^2 + 0.064T - 0.76} + \frac{t}{-0.0072T^2 + 0.64T - 0.59}} \quad (8)$$

The empirical Equation (8) represents the evolution model of Y_{TFn} during extraction of flavonoids from RGP with aqueous LA (0.5% *w*/*v*) and provides the values for Y_{TFn} at any time t and any temperature T, ranging between 10 and 320 min, and 30 and 60 °C, respectively.

3.2. Experimental Fitting (Model Validation)

A series of nine combinations of T and t were used to test the validity of the model in predicting Y_{TFn} values (Table 2). The observed and the predicted values were then analyzed by linear regression to ascertain the degree of correlation (Figure 3). It was found that the observed and the predicted values were highly correlated ($R^2 = 0.88$, $p = 0.0002$), suggesting that under the given experimental conditions, Y_{TFn} can be calculated with high reliability as a function of T and t, using Equation (8). The highest Y_{TFn} predicted was 13.27 mg·RtE·g^{-1} dpw, achieved at 40 °C, after 320 of extraction. The tendency in Y_{TFn} recorded was given in the form of a three-dimensional plot (Figure 4).

Table 2. Observed and predicted values of Y_{TFn} for a number of runs performed to assess the validity of the established extraction model.

Run	t (min)	T (°C)	Y_{TFn} (mg·RtE·g^{-1}·dpw)	
			Observed	Predicted
1	20	30	2.64	5.10
2	160	30	9.63	10.35
3	320	30	9.50	11.17
4	20	40	6.34	5.87
5	160	40	11.81	11.61
6	320	40	11.60	12.48
7	20	60	4.19	2.99
8	160	60	10.07	8.67
9	320	60	9.70	10.03

Figure 3. Correlation between the observed Y_{TFn} values and those predicted by the established kinetic model.

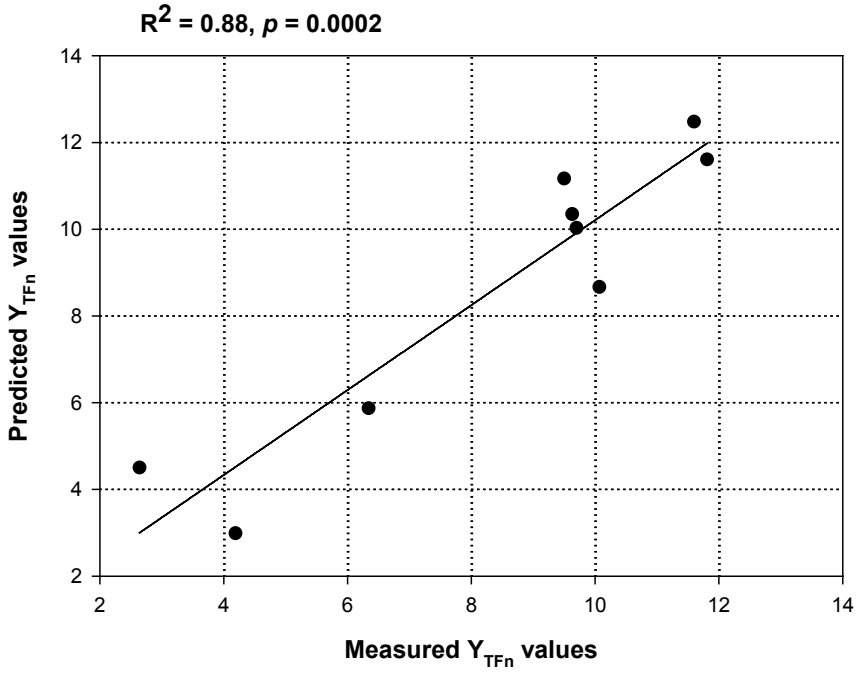
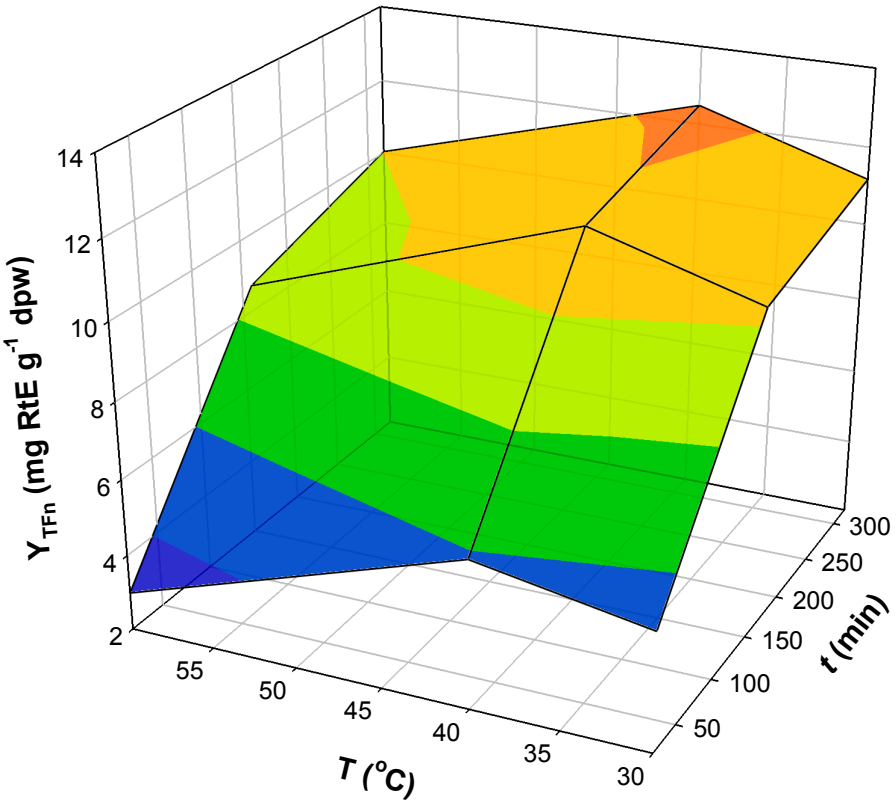


Figure 4. Three-dimensional plot illustrating the Y_{TFn} tendency of RGP extraction with 0.5% (*w*/*v*) aqueous LA, as a function of *t* and *T*.



3.3. Polyphenolic Composition

In order gain insight into the polyphenolic profile, the richest extract obtained (40 °C, 320 min) was analyzed by liquid chromatography-diode array-mass spectrometry (Table 3). Based on previously published data, Peaks 1 and 2 were tentatively identified as flavanol monomers and Peaks 6 and 7 as flavanol dimers [14]. In the same fashion, Peaks 3 and 5 were tentatively assigned to quercetin derivatives [14], while Peak 4 to an acylated cyanidin derivative [15].

Table 3. Spectral characteristics of the polyphenol tentatively identified in the RGP extract obtained with 0.5% (*w*/*v*) LA, after 320 min at 40 °C.

Peak #	Rt (min)	UV-Vis	$[M + H]^+$	Other Ions	Tentative Identity
1	9.69	280	291	-	(Epi)catechin
2	13.80	280	291	-	(Epi)catechin
3	21.64	256, 362	479	303	Quercetin 3-*O*-glucuronide
4	21.97	330, 506	595	-	Cyanidin 3-*O*-*p*-coumaroylglucoside
5	25.22	254, 360	464	303	Quercetin 3-*O*-glucoside or galactoside
6	40.99	240, 272	579	301	(Epi)catechin-(epi)catechin dimer
7	42.31	240, 272	579	301	(Epi)catechin-(epi)catechin dimer

Cyanidin-based pigments are not frequently encountered in RGP extracts, as several studies demonstrated the most abundant pigments to be peonidin 3-*O*-glucoside, malvidin 3-*O*-glucoside and malvidin 3-*O*-glucoside *p*-coumarate [15–18]. On the other hand, flavanols, such as catechin, epicatechin and dimers thereof [19–21], as well as flavonols, including myricetin, quercetin, kaempferol, quercetin 3-*O*-glucoside, quercetin 3-*O*-glucuronide and isorhamnetin 3-*O*-glucoside [22,23], are rather common RGP constituents. However, the identification of the major constituents of the extract (Table 3) revealed a different profile, evidencing a possible selectivity by the LA solution used for the extraction. It can be hypothesized that major substances, such as malvidin 3-*O*-glucoside, could not be extracted, because of the increased polarity of the LA solution. On the other hand, the most polar cyaniding 3-*O*-glucoside was the major pigment of the extract generated. This finding could be particularly important, as the addition of LA could modify a medium's polarity towards the extraction of specific metabolites.

The production of extracts containing a cocktail of the compounds reported above may have a significant prospect in industrial applications, such as the manufacturing of nutraceuticals and cosmetics, since almost all of these polyphenols have been claimed to possess beneficial biological activities [24]. In such a framework, the utilization of LA as a co-solvent in extracting functional polyphenols merits further investigation, mainly for the aspects pertaining to yield and selectivity. In this regard, it is proposed that future studies should embrace examinations in the direction of testing LA solutions as efficient extracting media using various plant materials and acid concentration/extraction time/temperature combinations.

4. Conclusions

Flavonoid recovery from grape pomace using aqueous LA was shown to be affected by both *T* and *t*. The course of the extraction was approached by the determination of basic kinetic parameters, which permitted the building of a predictive model. Using this model, the yield of flavonoid extraction, as a function of both *T* and *t*, could be very reliably estimated, under the experimental conditions deployed.

This study showed that the extraction of bioactive substances from RGP is not favored at temperatures over 40 °C. This is particularly important in developing an efficient process, where maximum recovery is always sought. Further, the LC-MS analyses demonstrated that the major phytochemicals recovered were flavonoids belonging to the flavanol, flavonol and anthocyanin classes. It is anticipated that similar studies might be of significant value in engineering extraction processes using environmentally-benign and food-compatible solvent systems, involving LA in combination with a solvent less polar than water (e.g., ethanol). Currently, work is in progress to investigate the effect of other critical parameters affecting the extraction process, such as non-toxic co-solvents, to further improve recovery yield.

Author Contributions

Katerina Tzima, laboratory experiments and data handling; Stamatina Kallithraka, preparation of the RGP, laboratory analyses; Yiorgos Kotseridis, preparation of the RGP, laboratory analyses; Dimitris P. Makris, experimental design, data handling, liquid chromatograph-mass spectrometry analyses, statistics.

Nomenclature

C_{TFn}	total flavonoid concentration (mg·RtE·L^{-1})
dpw	dry pomace weight (g)
h	initial extraction rate (mg·g^{-1}·min^{-1})
k	extraction rate constant (g·mg^{-1}·min^{-1})
T	temperature (°C)
t	time (min)
Y_{TFn}	extraction yield in total flavonoids (mg·RtE·g^{-1}·dpw)
$Y_{TFn(s)}$	extraction yield in total flavonoids at saturation (mg·RtE·g^{-1}·dpw)

Abbreviations

LA	lactic acid
RGP	red grape pomace
RtE	rutin equivalents

Conflicts of Interest

The authors declare no conflict of interest.

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