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Original Article

Optimization of the molybdenum blue method for estimating the antioxidant activity of natural products

[Optimización del método del azul de molibdeno para estimar la actividad antioxidante de productos naturales]

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Abstract

Context: The present experimental conditions of the molybdenum blue spectrophotometric method, used for antioxidant activity estimation, promote the degradation of flavonoids with potential interferences in the above determination.

Aims: To evaluate the effects of physicochemical factors on the formation of the complex for optimizing the total antioxidant activity method to better estimate the antioxidant activity of natural products.

Methods: A 3^{4-1} fractional experimental design was applied. Independent variables were temperature, color development time, type of acid and acidity, and the dependent variable was the absorbance of the complex. The concentration of ammonium molybdate tetrahydrate and sodium hydrogen phosphate remained constant throughout the study. The effects of the reducing agent and its concentration were studied independently. The effect of acidity in a wide range of values, the color development time considering temperature, the influence of co-solvents, and the antioxidant activity of various natural metabolites were also evaluated.

Results: Acid concentration and temperature greatly influenced the complex formation, making the type of acid and incubation time less significant. Ascorbic acid showed a shorter color development time than reference metabolites. Ethanol negatively influenced the amount of complex formed. The proposed conditions for developing this method were: type of acid, HCl or H_2SO_4; acid concentration 0.01 N; incubation temperature 65°C; and incubation time 40 min. Under these experimental conditions, the ranking order of antioxidant activity was pyrogallol>quercetin>ascorbic acid>gallic acid>rutin.

Conclusions: The new experimental conditions for the molybdenum complex assay give a more reliable determination of the antioxidant activity of natural products.

Keywords: antioxidants; flavonoids; molybdenum blue; research design.

Resumen

Contexto: Las condiciones experimentales actuales del método espectrofotométrico del azul de molibdeno, utilizado para la estimación de la actividad antioxidante, favorecen la degradación de los flavonoides con potenciales interferencias en la determinación anterior.

Objetivos: Evaluar los efectos de los factores fisicoquímicos en la formación del complejo para optimizar el método de actividad antioxidante total para una mejor estimación de la actividad antioxidante de los productos naturales.

Métodos: Se aplicó un diseño experimental fraccionado 3^{4-1}. Las variables independientes fueron la temperatura, el tiempo de desarrollo del color, el tipo de ácido y la acidez, y la variable dependiente fue la absorbancia del complejo. La concentración de molibdato amónico tetrahidratado y de hidrogenofosfato sódico se mantuvo constante durante todo el estudio. Los efectos del agente reductor y su concentración se estudiaron de forma independiente. También se evaluó el efecto de la acidez en un amplio rango de valores, el tiempo de desarrollo del color considerando la temperatura, la influencia de los co-solventes y la actividad antioxidante de varios metabolitos naturales.

Resultados: La concentración de ácido y la temperatura influyeron en gran medida en la formación de complejos, siendo menos significativos el tipo de ácido y el tiempo de incubación. El ácido ascórbico mostró un tiempo de desarrollo del color más corto que los metabolitos de referencia. El etanol influyó negativamente en la cantidad de complejo formado. Las condiciones propuestas para desarrollar este método fueron: tipo de ácido, HCl o H_2SO_4; concentración de ácido 0,01 N; temperatura de incubación 65°C; y tiempo de incubación 40 min. En estas condiciones experimentales, el orden de clasificación de la actividad antioxidante fue pirogalol>quercetina>ácido ascórbico>ácido gálico>rutina.

Conclusiones: Las nuevas condiciones experimentales para el ensayo del complejo de molibdeno proporcionan una determinación más fiable de la actividad antioxidante de los productos naturales.

Palabras Clave: antioxidantes; azul de molibdeno; diseño experimental; flavonoides.

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Abbreviations: DOE: Design of Experiment; SAR: Structure-activity relationships; TAA: Total Antioxidant Activity.

INTRODUCTION

The versatile nature of polyoxometalate compounds in terms of structure, size, redox behavior, photochemistry, and charge distribution, among other aspects, has made it one of the fastest-growing inorganic chemistry fields with many applications (Bijelic et al., 2019; Cherevan et al., 2020, Gavrilova et al., 2020; Liu and Wang, 2020; Myachina et al., 2021). All systems known as "molybdenum blue" are polyoxometalates. Many factors can affect the formation and chemical structure of these complexes (Long et al., 2007). Molybdenum blue, as a spectrophotometric method, has been widely used to estimate the antioxidant activity of natural products (Anokwah et al., 2022; Nazarudin et al., 2022; Patel and Ghane, 2021; Popoola, 2022; Purewal et al., 2022). The method is usually applied under the conditions described by Prieto et al. (1999), briefly: high acid concentration (H_2SO_4 0.6 M), high temperature (95°C), and long incubation time (90 min).

Phenols and flavonoids are secondary metabolites present in natural plant products with antioxidant properties, an aspect that is largely influenced by the chemical structure (Ali et al., 2013; Ami et al., 2007; Banjarnahor and Artanti, 2014). It is well known that flavonoids undergo degradation due to the influence of some factors, especially temperature and catalysis (acidic or basic) (Chaaban et al., 2017; Echeverry et al., 2018; Qiao et al., 2014; Ramešová et al., 2011; Wang and Zhao, 2016). The most significant is the fact that the degradation products interfere with the antioxidant activity, which can remain unchanged, increase, or decrease with respect to the original molecule (Chaaban et al., 2017). The stress conditions under which the molybdenum blue method is usually applied can cause an incorrect evaluation of the antioxidant activity of natural metabolites due to the interference of degradation products. Therefore, this work aimed to evaluate the effects of physicochemical factors on the formation of the molybdenum blue complex for optimizing the total antioxidant activity (TAA) method for a better estimation of the antioxidant activity of natural products.

MATERIAL AND METHODS

Materials, chemicals, and solvents

Ascorbic acid, gallic acid, and pyrogallol were standards purchased from Sigma-Aldrich®. Quercetin and rutin were from Acros Organics® and ammonium molybdate tetrahydrate $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ from Reachim®. Glacial acetic acid and ethanol were of spectroscopic grade from Acros Organics®. All other reagents were of analytical grade.

Experimental Design (DOE)

A 3^{4-1} fractional experimental design was carried out (four factors at three levels), consisting of 27 randomized experiments to determine the most significant factors. Independent variables were selected as temperature, color development time, type of acid, and acidity (expressed as normality, N) (Table 1). The reducing agent and its concentration remained unchanged, as well as the concentration of $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ and sodium hydrogen phosphate (Na_2HPO_4).

General procedure for DOE

A reactive mixture consisting of 1.0 mL of acid (acetic, sulfuric or hydrochloric acid), 0.7 mL of Na_2HPO_4 (28 mM) and 0.7 mL of $(NH_4)_6Mo_7O_{24}.4H_2O$ (4 mM) was prepared; then a 0.6 mL of the reducing agent was added (ascorbic acid, final concentration 0.15 mM) to initiate the formation of the molybdenum blue complex. All reagents were prepared in distilled water. The samples were incubated at different times and temperatures according to the designed factor analysis; similarly, the type of acid and the final acidity of the solutions were changed (Table 1). After cooling, the absorbance of the samples was measured at 695 nm in UV-Vis spectrophotometer (UV-1601, Beijing Rayleigh Analytical Instruments Corp., China), to evaluate the amount of molybdenum blue complex formed.

Table 1. Experimental design.

Factors	Levels		
	Low	Medium	High
Temperature (°C)	40	65	90
Type of acid	CH_3COOH	H_2SO_4	HCl
Time (min)	15	60	120
Acidity (N)	0.0025	0.025	0.25

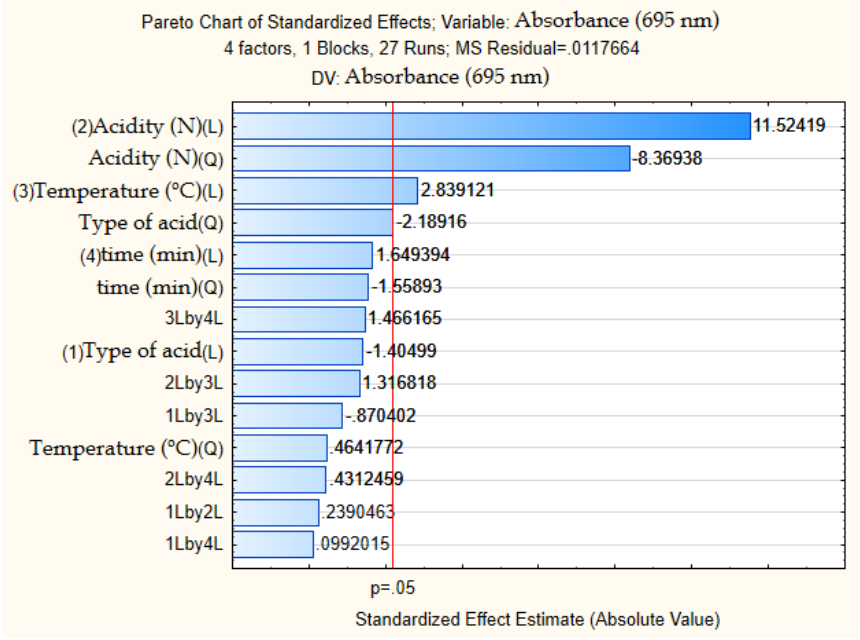

Figure 1. Pareto chart.

Both acid concentration (N) and temperature (°C) show strong effects on the molybdenum blue complex formed.

Independent experiments to define the optimal conditions to develop the spectrophotometric method

After obtaining the results from DOE, the following were studied in greater depth: a) the effect of acidity in a wide range of values (0.0025 N to 1 N); b) the color development time (0 min to 60 min) considering temperature (45, 55, 65, and 90°C); c) the influence of co-solvents (ethanol); and d) the antioxidant activity of various natural metabolites was evaluated, and the correspondence of the results with the structure-activity relationships (SAR) reported for the antioxidant activity of polyphenols was analyzed.

In the experiment where the effect of acidity on the formation of the molybdenum blue complex was evaluated, the reaction mixture was performed in water due to the great solubility of the ascorbic acid used as a reducing agent. When the influence of co-solvents (ethanol) in the formation of the complex was evaluated, ascorbic acid was used as a reducing agent, according to the general conditions described above, but in an experimental group, an equivalent volume of ethanol replaced 0.3 mL of distilled water. In the other experiments, considering the limited solubility of the flavonoids quercetin and rutin, ethanol was used as a co-solvent. In this case, the flavonoids were previously dissolved in ethanol, and then 0.3 mL of each solution was added to the reaction mixture. To guarantee the homogeneity of the resulting hydro-alcoholic mixture, even when testing substances are highly soluble in water (e.g., gallic acid or pyrogallol), a volume of 0.3 mL of ethanol was always added.

Statistical analysis

Statistical analysis was performed using the software package Statistica 7.0, StatSoft Inc., USA. A 3^{4-1} factorial analysis was applied. The analysis of the results was carried out through a Pareto chart and a surface-response graph for the factors with the greatest influence. One-way ANOVA with subsequent Duncan's test was applied to find significant differences in the amount of molybdenum blue complex formed in the different set conditions. The level of significance was defined as $p<0.05$.

RESULTS

Influence of acidity, temperature, and incubation time on molybdenum blue complex formation

Acidity (expressed as normality, N) and temperature were the most important factors in the formation of molybdenum blue (Fig. 1).

The fitted model, expressed in equation [1], describes the formation of the complex:

$$Abs = -0.21524319740618 + 22.824142661182x - 80.96770170845x^2 + 0.00252y \quad [1]$$

Where Abs: Absorbance [amount of molybdenum blue complex formed]; x: acidity (N); y: temperature (°C).

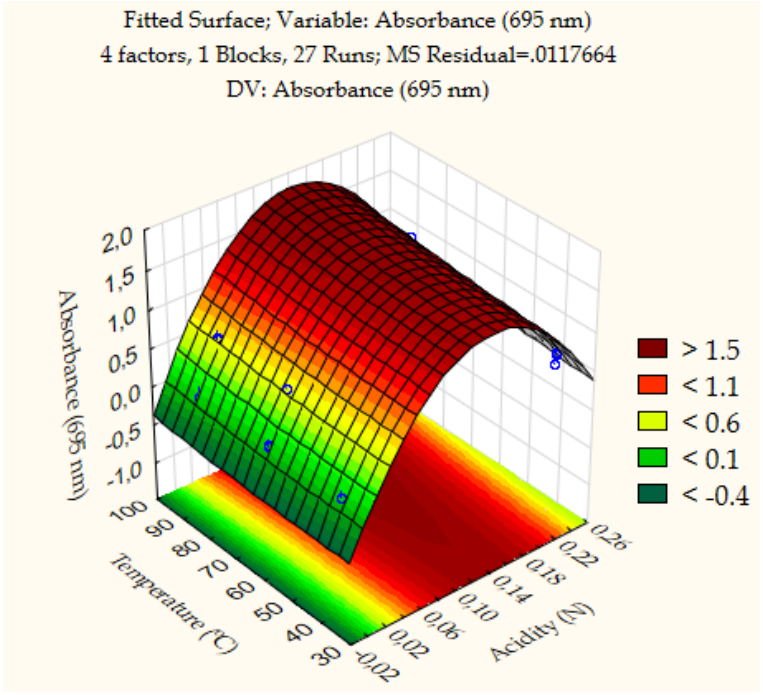

Figure 2. Response surface estimated. A quadratic relationship between acidity and the amount of molybdenum blue complex was found.

Acidity strongly influences the formation of the molybdenum blue complex. Over a wide range, it contributes positively to the complex formation, but high values influence it negatively; presumably, the strong acid medium affects the reducing agent (Fig. 2).

A more detailed analysis of the influence of acidity in the 0.0025 N - 1 N range (pH ≈ 5 - 1) reveals that the formation of the complex was significant when the acidity was set at 0.01 N (pH ≈ 3). An increase in acidity above 0.01 N does not imply any advantage in the formation of the complex. It remains unchanged (0.01 N to 0.1 N range, Duncan test, $p>0.05$), increases slightly (0.25 N – 0.5 N range, Duncan test, $p<0.05$), or drastically decreases when it reaches the value of 1 N (Duncan test, $p<0.05$) (Fig. 3).

Considering these results, the choice of acidity of 0.01 N (HCl or H_2SO_4) is recommended since it allows an adequate formation of the molybdenum blue complex and reduces the risk of acid catalysis of natural products. The negative effect of a very strong acidic medium on the formation or stability of molybdenum blue complexes has been previously reported (Iorio et al., 1991).

An important aspect of choosing a spectrophotometric method for estimating antioxidant activity is the time required to perform the readings. Although the results of the factorial design revealed that incubation time was not an important factor, it must be considered that the interval evaluated was from 15 min to 90 min. This suggests that after 15 min, the amount of molybdenum blue complex, when ascorbic acid is used as a reducing agent, did not vary noticeably. However, additional studies are required to determine the minimum time to perform the spectrophotometric determination, considering, in turn, the influence of temperature on the color development time.

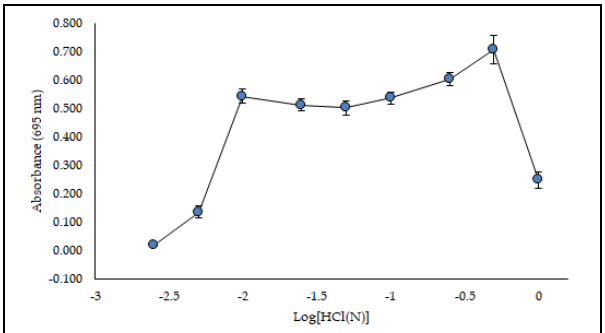

Figure 3. Effect of acidity on the formation of the molybdenum blue complex when the reducing agent was ascorbic acid 0.15 mM, the type of acid was HCl, and the acidity range 0.0025 N – 1 N.

Results are expressed as arithmetic mean ± SD (n = 5).

The color development time showed a similar behavior at different temperatures, and the absorbance readings were stable from 10-15 min (Fig. 4). However, small differences were observed depending on the incubation temperature. When incubated at the low-

est temperature, 45°C, the minimum time required to obtain a stable reading was 15 min, verifying that the amount of complex formed was significantly higher compared to incubation times of 5 min (0.509 AU ± 0.047 AU *vs.* 0.249 AU ± 0.092 AU, Duncan test, $p<0.05$) and 10 min (0.509 AU ± 0.047 AU *vs.* 0.450 AU ± 0.060 AU, Duncan test, $p<0.05$) respectively. A further increase in incubation time (15 min – 60 min) at this temperature did not significantly increase the amount of complex formed (Duncan test, $p>0.05$). At higher temperatures (55–90°C), the time required for molybdenum blue complex formation was similar, and the stable reading was reduced to 10 min (Duncan test, $p<0.05$). Considering the above, under the conditions described, the minimum time to perform the spectrophotometric determination was 10 min when ascorbic acid (0.15 mM) was used as a reducing agent.

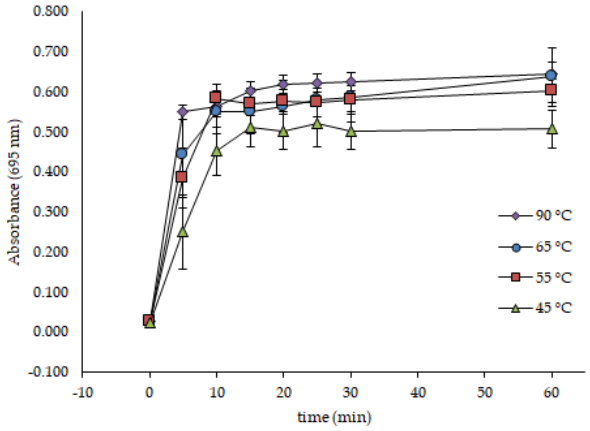

Figure 4. Influence of temperature on color development time when reducing agent was ascorbic acid 0.15 mM and acid/acidity of HCl 0.01 N.

Results are expressed as arithmetic mean ± SD (n = 7).

The temperature, on the other hand, generally increased the total amount of molybdenum blue complex (Fig. 4), but the influence of this factor was much less than the acidity of the medium. Considering the results obtained at 15 min of incubation, the amount of molybdenum blue complex at 55°C did not show significant differences compared to 65°C (0.570 AU ± 0.032 AU *vs.* 0.550 AU ± 0.052 AU; Duncan test, $p>0.05$) and 90°C (0.570 AU ± 0.032 AU *vs.* 0.601 AU ± 0.023 AU, Duncan test, $p>0.05$) respectively. This implies that a temperature increase from 55 to 90°C is unnecessary and increases energy consumption and the risk of degradation reactions favored by high temperatures. However, the amount of complex formed at this temperature (55°C) was significantly higher than that formed at an incubation temperature of 45°C (0.570 AU ± 0.032 AU *vs.* 0.509 AU ± 0.047 AU; Duncan test, $p<0.05$). This indicates that although a low temperature limits the formation of the molybdenum blue complex, an increase in this factor above 55°C does not provide any advantage and only contributes to energy expenditure and the risk of thermal stress.

Interestingly, when quercetin was used as a reducing agent, the results greatly differed from those obtained with ascorbic acid (Fig. 5).

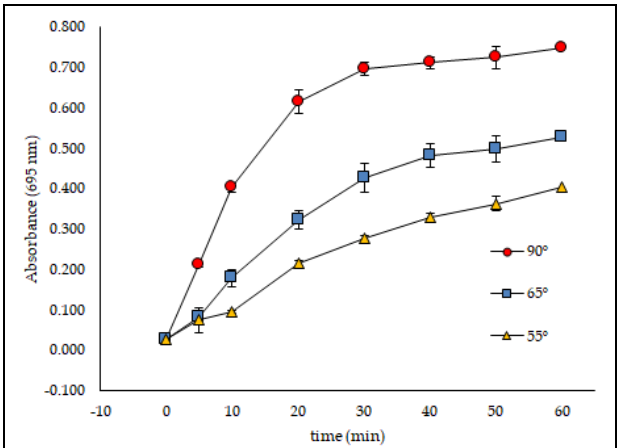

Figure 5. Influence of temperature on color development time when reducing agent was quercetin 0.1 mM and type of acid/acidity of HCl 0.01 N.

Results are expressed as arithmetic mean ± SD (n = 7).

The color development time was longer for the flavonoid, and the temperature greatly influenced the amount of molybdenum blue complex. This is possibly due to the degradation products present in the medium and the effect of temperature on the reducing power of the flavonoid. Unlike what was observed in the experiments where ascorbic acid was used as a reducing agent, absorbance readings were not stable in the range of 5-60 min at 55°C. On the other hand, when the incubation temperature was 65°C, the color development time was 40 min. At this temperature, the amount of molybdenum blue complex increased significantly in a range from 5 min to 40 min (Duncan test, $p<0.05$), obtaining stable readings from 40 to 60 min of incubation (Duncan test, $p>0.05$). An increase in temperature to 90°C caused an increase in the amount of molybdenum blue complex (0.711 AU ± 0.017 AU *vs.* 0.481 AU ± 0.036 AU, $p<0.05$), and the color development time was reduced to 30 min. However, considering that flavonoids (such as quercetin, rutin, luteolin, and many others) can undergo thermal degradation, and that is generally very significant when the temperature increases above 70°C (Chaaban et al., 2017), as well as the possible contribution of acid catalysis, the use of such high temperature is inappropriate to favor the increase in the amount of molybdenum blue complex formed.

As previously discussed, these results could be related to the presence of degradation products, which may have greater antioxidant activity than the initial product. Considering the behavior of the quercetin, a minimum incubation time required of 40 min is necessary when evaluating the antioxidant activity of natural products. The preferable incubation temperature would be 65°C, which allows the shortest color development time with the greatest amount of molybdenum blue complex formed, but avoiding possible degradation reactions due to the combined effects of temperature and acid medium

Effect of ethanol on the formation of the molybdenum blue complex

Many natural products have limited solubility in water, which in the case of flavonoids is much lower when they are not glycosylated or have a smaller number of hydroxyl groups in their structure. This requires the use of co-solvents such as ethanol. However, the presence of organic solvents is one of the factors that can influence the formation of polyoxometalate complexes (Long et al., 2007). When the effect of ethanol on the amount of molybdenum blue complex formed was evaluated, a statistically significant decrease was observed for the samples incubated at 65°C/40 min (0.613 AU ± 0.02 AU *vs.* 0.570 AU ± 0.02, ANOVA test, $p<0.05$), and although the difference is not very large, it must be considered during the evaluation of metabolites that require the use of this co-solvent (Fig. 6).

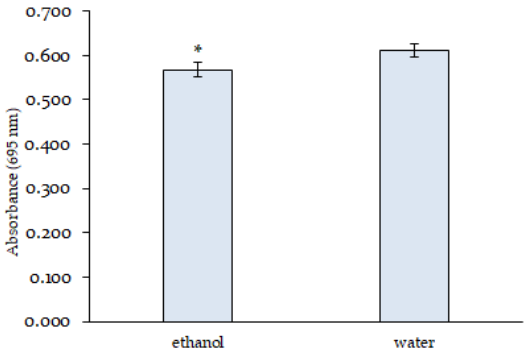

Figure 6. Effect of ethanol (co-solvent) on the amount of molybdenum blue complex formed when reducing agent was ascorbic acid 0.15 mM; acid/acidity: HCl, 0.01 N at 65°C/40 min.

Results are expressed as mean ± SD (n = 5), *ANOVA, $p<0.01$.

Evaluation of the antioxidant activity of natural metabolites under the selected conditions

The possibility that a phenolic compound donates electrons and acts as a reducing agent (antioxidant) is related to the stability of the phenoxy radicals formed. The number, position, and functionalization (e.g., O-glycosylation, O-methylation) of the phenolic hydroxyl groups (present in phenols and flavonoids), the effects of electron donor (e.g., NH_2) or electron acceptor (e.g., NO_2) groups present, in addition to other structural aspects, determine the antioxidant activity of a particular phenolic compound (Ali et al., 2013; Ami et al., 2007; Banjarnahor and Artanti, 2014).

Pyrogallol (benzene-1,2,3-triol), a polyphenol structurally related to gallic acid (3,4,5-trihydroxybenzoic acid), showed the highest antioxidant activity (Fig. 7). The structural differences between these metabolites explain this behavior. Gallic acid has a carboxyl group (electron-withdrawing group) that decreases the stability of phenoxy radicals and, therefore, their antioxidant activity. On the other hand, quercetin also showed great activity; however, glycosylation of the 3-hydroxyl group (Fig. 8), present in rutin, completely inhibits the antioxidant power in the same concentration range.

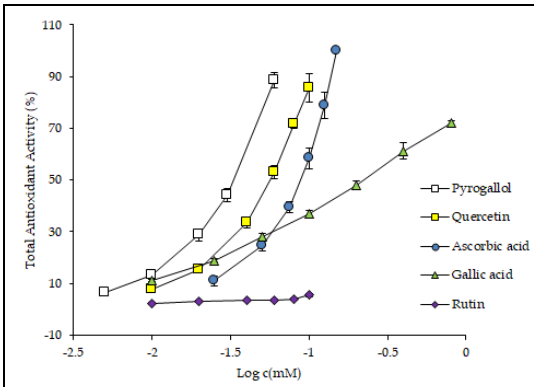

Figure 7. Total antioxidant activity (TAA) of some plant metabolites based on the proposed experimental conditions.

Results are expressed as mean ± SD (n = 5); Concentration (0.005–0.8 mM) on a logarithmic scale. [1]TAA 100 %: Amount of complex formed due to the addition of ascorbic acid 0.15 mM and HCl 0.01 N at 65°C/40 min; λ = 695 nm.

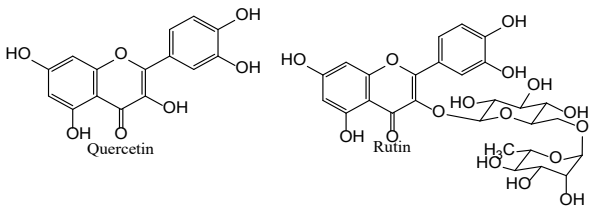

Figure 8. Chemical structure for the flavonoid quercetin and its 3-O-glycosylated derivative, rutin.

Source: Drawing from authors using software ChemDraw, 2014.

These findings are consistent with reported SAR studies for the antioxidant activity of flavonoids and phenols (Ali et al., 2013; Ami et al., 2007; Banjarnahor and Artanti, 2014), so that the modifications developed to the spectrophotometric method do not lead to errors in estimating the antioxidant activity of these natural products. Under these experimental condi-

tions, the ranking order of antioxidant activity for the evaluated products was as follows: pyrogallol>quercetin>ascorbic acid>gallic acid>rutin (Table 2).

Table 2. EC_{50} values calculated for some antioxidant products using the proposed experimental conditions.

Antioxidant	EC_{50} (mM)
Pyrogallol	0.034 ± 0.002*
Quercetin	0.058 ± 0.002*
Ascorbic acid	0.085 ± 0.004*
Gallic acid	0.211 ± 0.015*
Rutin	ND

Results are expressed as mean ± SD (n = 5). ND-not determined, *significant differences with respect to all groups (Duncan test, p<0.05).

Study limitations

It is possible to scale the method to microplate readings, which reduces the amounts of reagents to use and increases the speed of determinations by simultaneously evaluating various plant metabolites or natural products. In fact, the blue molybdenum method adapted for microplate readings has been previously reported for the evaluation of the antioxidant activity of natural products (Asghar et al., 2022) but under the usual conditions that could promote the degradation of plant metabolites.

CONCLUSION

Considering the results, the suggested conditions for developing the TAA spectrophotometric method are acidity (HCl or H_2SO_4) 0.01 N (pH~3), incubation temperature of 65°C, and incubation time of 40 min. The addition of ethanol (co-solvent) to the reaction mixture slightly decreased the formation of the molybdenum blue complex, but it can be used as a co-solvent to promote the solubility of less polar flavonoids. The optimized experimental conditions for the molybdenum blue complex assay give a more reliable determination of the antioxidant activity of natural products.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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AUTHOR CONTRIBUTION:

Contribution	Hernández-Barreto E	Ruz-Sanjuan V	Ribalta-Ribalta V	Torres-Gómez LA
Concepts or ideas	x	x	x	x
Design	x	x	x	x
Definition of intellectual content	x	x	x	x
Literature search	x		x	
Experimental studies	x		x	
Data acquisition	x		x	
Data analysis	x	x		x
Statistical analysis	x	x		x
Manuscript preparation	x	x		
Manuscript editing	x	x		
Manuscript review	x	x	x	x

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