

# Extraction of Kaempferitrin and Astragalín from *Justicia Spicigera* by Supercritical Fluid Extraction and Its Comparison with Conventional Extraction

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**Abstract:** A study of supercritical fluid CO<sub>2</sub> extraction of kaempferitrin (KM) and astragalín (KG) from *Justicia spicigera* (muicle) was conducted. A 3<sup>3</sup> Box-Behnken design was used to analyze the effects of pressure (200-300 bar), temperature (40-60° C), and co-solvent flow rate (0.5-1.0 mL/min). The highest KM and KG concentration were achieved at a pressure of 300 bar, a temperature of 60° C, and co-solvent flow rate of 1.0 mL/min (ethanol 99.5 %), with a constant CO<sub>2</sub> flow rate of 5 mL/min and extraction time of 180 min. Under these conditions, the experimental values for KM and KG (115.08±2.81 and 56.63±9.02 mg/100 g of dry powder, respectively) were similar to those calculated by the models (109.0 and 44.07 mg/100 g of dry powder, respectively). The use of 70 % ethanol as co-solvent in the supercritical extraction process considerably improved the yields of KM and KG (562.71±156.85 and 79.90±18.03 mg/100 g of dry powder, respectively) compared to the 99.5 % ethanol extractions. The conventional extraction showed the highest yields of KM and KG (574.20±65.10 and 113.10±15.06 mg/100 g of dry powder, respectively) at 70° C and extraction time of 120 min. Adequate yields were achieved of KM and KG by supercritical fluid extraction compared with conventional extraction (98 and 70 %, respectively); therefore supercritical fluid extract of *J. spicigera* could be used in the development of functional foods, as well as its possible use in traditional medicine by the health professionals.

**Keywords:** Astragalín; *Justicia spicigera*; Kaempferitrin; Supercritical fluid extraction.

## 1. Introduction

The use of medicinal plants in alternative medicine to treat various ailments or diseases (respiratory, circulatory, digestive, genitourinary, musculoskeletal and nervous systems; endocrine, nutritional and metabolic diseases; injuries and intoxications; infectious and parasitic diseases; mental and behavioral disorders; skin and subcutaneous tissue disorders; neoplasms, carcinoma, and cancer), continues to be a deeply rooted practice in Mexico [1-3]. In this regard, one of these important plants is *Justicia spicigera* (known as “muicle” or “muitle”), an endemic plant from Mesoamerica that grows in Mexico, Central America, and some areas of the United States. In Mexico, it has been used since pre-Hispanic times to treat dysentery, gonorrhea, scabies, fever and uterine bleeding [4]. Currently, it is still used for medicinal purposes to treat cancer, circulatory diseases, diabetes, leukemia, anemia, giardiasis, fascioliasis, diarrhea, nerves, rheumatism, stomach inflammation and headache [4, 5]. Several research shows that the extract (aqueous, ethanolic and methanolic) of *J. spicigera* leaves have antioxidant [6], antimicrobial [7], anticancer [8], antidiabetic [9], antiepileptic [10], and antidepressant properties [11]. These properties are due to the presence, mainly of phenolic and flavonoids compounds. *Justicia spicigera* extracts contain significant concentrations of flavonoids, mainly kaempferol glycosides, the most important are: kaempferitrin (kaempferol-3,7-dirhamnoside) and astragalín (kaempferol-3-β-D-glucopyranoside), of which, it has been proven their antidiabetic [12, 13], and anticancer [3, 14] properties. Several studies [15-18], has been investigated the extraction of these compounds using different extraction methods (ultrasound-assisted extraction, ultrasound-microwaves, microwaves, and simple extraction). However, there is little information about the extraction of kaempferol and its glycosides from *J. spicigera*, using supercritical fluids.

The use of supercritical fluid technology (mainly CO<sub>2</sub>) to extract bioactive compounds from plant sources, is having a very important increase due to quality and purity of the obtained extracts, and because it is a technology

that does not generate toxic waste. Furthermore, the process is performed at relatively low temperatures, in the absence of light and oxygen, which avoids degradation of labile compounds, as well as oxidative reactions [19]. One of the supercritical CO<sub>2</sub> disadvantages is its low polarity (it behaves like a lipophilic solvent), which results in a minimum extraction of polar compounds, mainly phenols, flavonoids, and terpenoids. To increase the polarity and selectivity of supercritical CO<sub>2</sub>, the use of co-solvents (mainly ethanol due to its low toxicity) has been suggested for significantly improve the extraction of these polar compounds [20, 21]. However, it has been reported [22-24] that some phenolic and flavonoid compounds (gallic acid, protocatechuic acid, caffeic acid, methyl gallate, catechin, rutin, myrecitin, quercetin, kaempferol) are actually soluble in supercritical CO<sub>2</sub> when pressures are high (above 200 bar), because fluid density increases, as the pressure increases at constant temperature. The aim of this work was to perform a study to obtain kaempferitrin and astragalins from *J. spicigera* by supercritical fluid CO<sub>2</sub> extraction, using ethanol as co-solvent and compare it with conventional extraction. To the best of our knowledge, this is the first application of supercritical fluid extraction to extract and recover these compounds from *J. spicigera* leaves.

## 2. Materials and methods

### 2.1 Sample preparation

Muicle leaves (*J. spicigera*) from the region of Cuautla, Morelos, Mexico were used (the leaves were harvested at the adult phase of the plant). The leaves were selected, washed, and dried at room temperature ( $31 \pm 1$  °C, relative humidity of  $75 \pm 7$  %) until the moisture content was below 10 %. The dried leaves were ground in a stainless steel sprayer mill (Veyco MPV 100, Mexico) with a mesh of 0.5 mm, the powder obtained was passed through a Tyler mesh 14 (1.4 mm opening sieve) and stored in sealed bags. The moisture content, water activity and average particle size were assessed.

### 2.2 Moisture content

It was measured according with the Mexican Official Standard [25]. The Sample was dehydrated at 100° C for 4 h in a convection oven (Boekel 107800, Boekel Industries Inc. Lower Southampton, PA, USA) at atmospheric pressure. The analysis was performed in triplicate.

### 2.3 Water activity ( $a_w$ )

It was measured using an AQUA-LAB hygrometer model 4TE (Decagon Devices Inc., Pullman, WA, USA) with internal temperature control ( $15-50 \pm 0.2$ ° C). The equipment was calibrated with standard solutions of 6.0 M NaCl ( $a_w = 0.76 \pm 0.003$ ) and 8.57 M LiCl ( $a_w = 0.50 \pm 0.003$ ) at  $25 \pm 0.1$ ° C. The analysis was performed in triplicate.

### 2.4 Average particle size analysis

The average particle size was performed using a particle analyzer with laser light diffraction Mastersizer 3000 (Malvern Instruments Limited, Worcestershire, UK) with a measuring range of 0.01 to 3500 µm. About 1 to 1.5 g of sample was used. The size distribution and cumulative weight curves were obtained, as well as the average diameter  $d_{50}$  [26, 27]. The analysis was performed in triplicate.

### 2.5 Supercritical fluid extraction (SFE)

It was performed by using the SFE unit shown in Figure 1 (equipment designed in the lab). The sample ( $5.01 \pm 0.01$  g) was placed in the sample-holder cell (9) and thereafter was placed in the extraction cell (10). The extraction cell was placed in a convection oven (12), the extraction temperature was set, and liquid CO<sub>2</sub> and the co-solvent (ethanol 99.5 %) were pumped into extraction cell after the desired temperature was achieved. Once extraction conditions (pressure and temperature) were achieved, the recovery cell valve (16) was opened keeping a supercritical CO<sub>2</sub> flow rate of 5 mL/min. The conditions were set as follows: pressure 200-300 bar, temperature 40-60° C and co-solvent flow rate of 0.5-1.0 mL/min. The extraction time was 180 min and at the end of this, liquid CO<sub>2</sub> and ethanol were pumped through the SFE unit until co-solvent did not show extract residues. Preliminary tests were performed in order to specify the extraction time (Fig. 2). The extracts were placed in Erlenmeyer flasks with screw cap (capacity 125 mL) covered with aluminum foil and kept at -15° C until HPLC analysis. The volume and weight of the extracts were measured.

### 2.6 Conventional extraction

The conventional extraction method was performed using  $2.52 \pm 0.01$  g of sample, which was diluted with 50 % (by mass) ethanol (0.1 g/mL) to make 25 mL in a volumetric flask [28]. The solutions were placed in test tubes with screw cap (capacity 50 mL) covered with aluminum. The extraction process was carried out using a 2x2 factorial design, two temperatures (50 and 70° C, in a water bath) and two extraction times (60 and 120 min).

During the extraction process, the solutions were shaken with a Vortex (G-560, Scientific Industries, Inc. Bohemia, N. Y. USA) at 15 min intervals [29]. The solutions were filtered through Whatman paper No. 4 and made up to a volume of 25 mL with distilled water. The extracts were placed in 50 mL flasks covered with aluminum foil and kept at  $-15\text{ }^{\circ}\text{C}$  until HPLC analysis. The extractions were performed in triplicate.

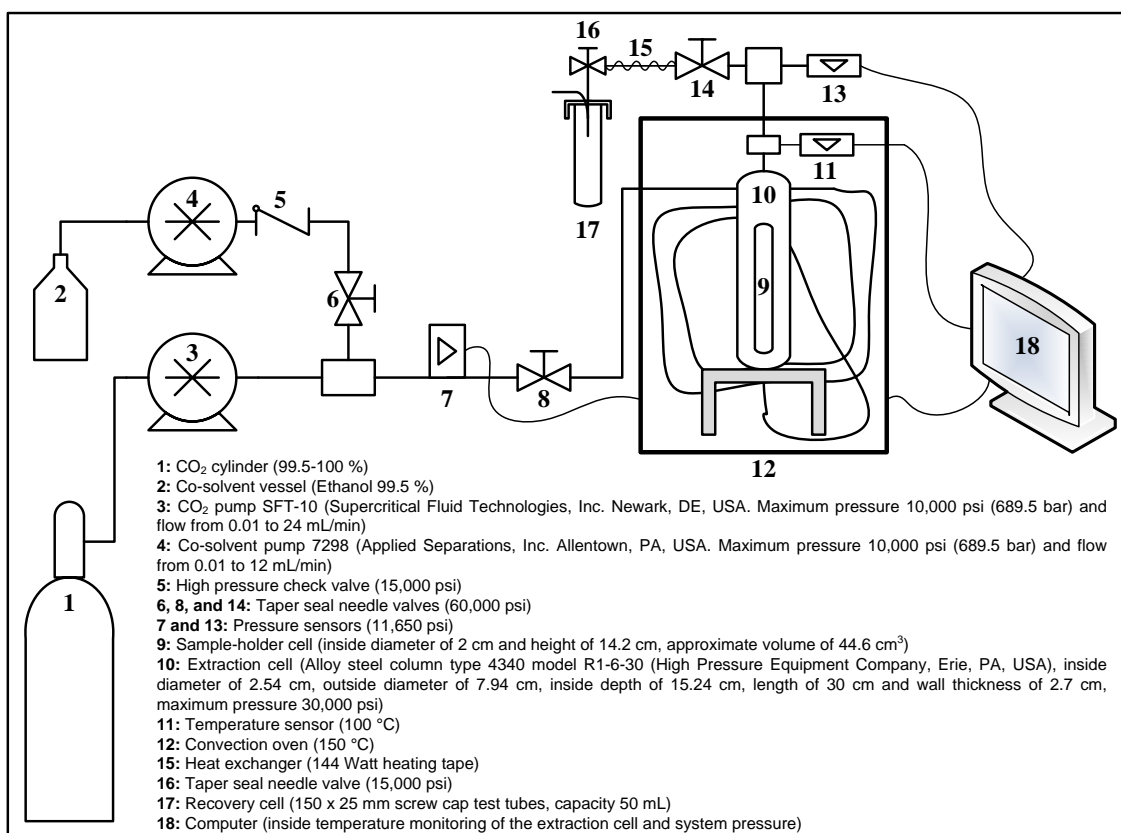


Figure 1. Schematic diagram of the supercritical fluid extraction (SFE) unit.

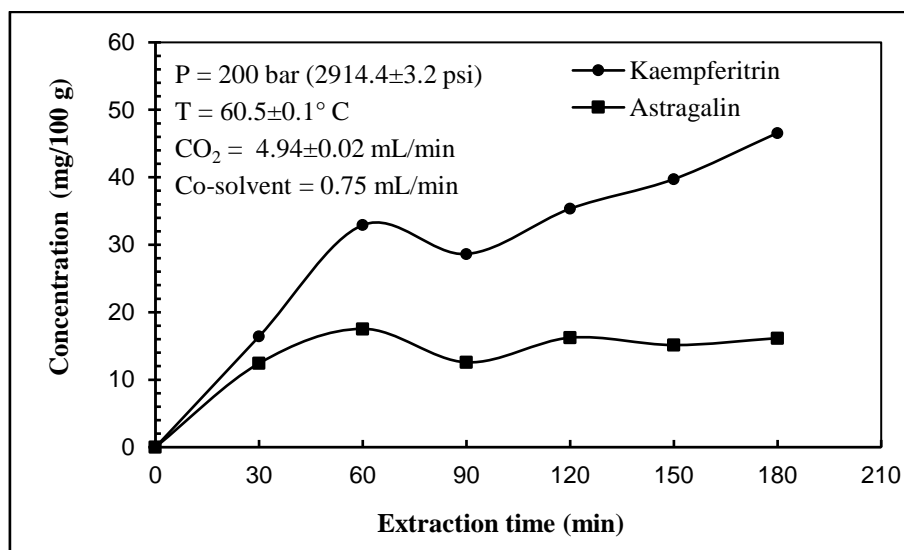


Figure 2. Kaempferitrin and astragalin concentration at different times by supercritical fluid CO<sub>2</sub> extraction and ethanol as co-solvent.

## 2.7 Identification and quantification of kaempferitrin (KM) and astragalin (KG) by HPLC analysis

A volume of 1 mL of the extracts was taken and diluted with methanol (HPLC grade 99.96 %, JT Baker) to 10 mL in a volumetric flask. Afterward, diluted extracts were filtered through a 0.45 μm membrane filter (Millipore)

and transferred into 1.2 mL HPLC vials and capped. The HPLC analysis was performed using a Waters 1525 HPLC (Milford, MA, USA) equipped with a binary pump system, Waters 2487 dual absorbance detector (190 to 700 nm) and Waters 717 autosampler (injection volume 0.1 to 2000  $\mu\text{L}$ ). A Dionex Acclaim® 120 C18 column (150 mm x 4.6 mm i. d.) packed with 5  $\mu\text{m}$  diameter particles was used. The extracts (10 and 200  $\mu\text{L}$ ) were eluted with acetic acid (A) (HPLC grade  $\geq 99.7\%$ , JT Baker) at 2 % and acetonitrile (B) (HPLC grade 99.98 %, JT Baker), at wavelength 265 and 368 nm [30], and according to the following elution gradient [31]: 90 % A and 10 % B, 0 - 2.0 min (flow rate: 1.0 mL/min); 55 % A and 45 % B, 2.0 - 6.4 min (flow rate: 1.0 mL/min); 45 % A and 55 % B, 6.5 - 7.0 min (flow rate: 1.0 mL/min); 30 % A and 70 % B, 7.1 - 7.4 min (flow rate: 0.8 mL/min); 20 % A and 80 % B, 7.5 - 8.0 min (flow rate: 0.2 mL/min); 30 % A and 70 % B, 8.1 - 9.0 min (flow rate: 0.5 mL/min); 90 % A and 10 % B, 9.1 - 16.0 min (flow rate: 1.0 mL/min). A volume of 10 and 200  $\mu\text{L}$  was injected of the conventional and supercritical fluid extracts, respectively. For quantification of KM (Kaempferol-3,7-dirhamnoside  $\geq 97\%$ , standard substance from Sigma) and KG (kaempferol-3- $\beta$ -D-glucopyranoside  $\geq 97\%$ , standard substance from Sigma), a solution (3 mL) was prepared with equal volumes (1 mL) of KM (0.28 mg/mL) and KG (0.34 mg/mL) using methanol (HPLC grade) as solvent. Of this solution, volumes of 2, 4, 6, 8 and 10  $\mu\text{L}$  were taken, equivalents to 0.19, 0.37, 0.56, 0.75 and 0.93  $\mu\text{g}$  for KM and 0.23, 0.45, 0.68, 0.91 and 1.13  $\mu\text{g}$  for KG, respectively. Results were expressed as milligrams of KM or KG per 100 g of dry sample.

## 2.8 Experimental design for optimization

In order to determine the optimal conditions of extraction process and in accordance to several researches [24, 32-35], a  $3^3$  Box-Behnken design without replicates (Table 1) was performed using response surface methodology (RSM), employing MINITAB® software. The independent variables were pressure (200-300 bar), extraction temperature (40-60° C) and co-solvent flow rate (0.5-1.0 mL/min, ethanol 99.5%), with a total 15 experiments including 3 central points. The extraction time and CO<sub>2</sub> flow rate were kept constant (180 min and 5 mL/min, respectively). The dependent variables were kaempferitrin (KM) and astragalín (KG) content. The experimental results were adjusted to a second-order polynomial model (Eq. 1):

$$Y = \beta_0 + \beta_1A + \beta_2B + \beta_3C + \beta_4A^2 + \beta_5B^2 + \beta_6C^2 + \beta_7AB + \beta_8AC + \beta_9BC \quad (1)$$

Where  $Y$  is the predicted response;  $A$  is the pressure;  $B$  is the extraction temperature;  $C$  is the co-solvent flow rate;  $\beta_0$  is the intercept;  $\beta_1, \beta_2, \beta_3$  are linear coefficients;  $\beta_4, \beta_5, \beta_6$  are quadratic coefficients;  $\beta_7, \beta_8, \beta_9$  are interaction coefficients. The model fit and the importance of the estimated parameters were determined by an analysis of variance (ANOVA). With the optimal conditions obtained in the supercritical extraction process (pressure, temperature, and co-solvent flow rate), the extraction of KM and KG was performed using 70 % ethanol as co-solvent [33] in order to compare the yields with those obtained by pure ethanol (99.5%), under the same conditions.

## 2.9 Statistical analysis

The effect of temperature and extraction time on the KM and KG content in conventional extracts of muicle, were analyzed by one-way analysis of variance (ANOVA) using Minitab (Release 17.1.0; Minitab Inc., State College, PA, USA). Differences were considered significant for values of  $p \leq 0.05$ .

## 3. Results and discussion

### 3.1 Granulometry and physic properties of powdered *Justicia spicigera* leaves

The granulometric distribution analysis of the powdered muicle leaves (*J. spicigera*) used for obtaining extracts, had an average diameter ( $d_{50}$ ) of  $638.3 \pm 76.9$   $\mu\text{m}$ , with a moisture of  $7.86 \pm 0.22\%$  and  $a_w$  of  $0.53 \pm 0.02$ .

### 3.2 Evaluation of the models' suitability

The results of all Box-Behnken design experiments are shown in Table 1. In the first evaluation, the analysis was performed considering all the terms of the mathematical model (Eq. 1). The quadratic term, the linear term and the cross-product terms between the pressure ( $A$ ), extraction temperature ( $B$ ) and co-solvent flow rate ( $C$ ), were considered statistically significant ( $p \leq 0.05$ ). In order to get a better model, only the significant terms were included in the model [36].

Table 2 shows the regression parameters for each response variable. It can be observed that the analysis of variance was statistically significant for the two models and the lack of fit was not significant ( $p > 0.05$ ), which indicates that the models could be adjusted properly to the experimental data. With respect to the coefficient of determination ( $R^2$ ), commonly is used to explain the overall predictive capability of the model. The values obtained from  $R^2$  were 0.755 and 0.876, which indicates the models fit well to the experimental data since values higher than 0.75 indicates aptness of the model [37]. However, a value close to 1 of  $R^2$  does not necessarily imply that the regression model be suitable, since adding a variable to the model will always increase  $R^2$ , regardless of

whether the additional variable is statistically significant or not. Therefore, it is possible for models that have large values of  $R^2$  to yield poor predictions of new observations or estimates of the mean response [38].

Table 1. Box-Behnken design and experimental data for the content of kaempferitrin (KM) and astragalins (KG).

Run	Independent variables			Dependent variables	
	Pressure (bar)	Temperature (°C)	Co-solvent flow rate (mL/min)	KM (mg/100 g)	KG (mg/100 g)
1	250	60	0.50	8.37	5.61
2	200	50	1.00	110.10	31.49
3	300	50	1.00	109.98	26.43
4	250	60	1.00	76.52	30.29
5	250	50	0.75	28.90	10.69
6	250	50	0.75	55.04	17.64
7	300	40	0.75	73.35	19.90
8	250	40	0.50	10.23	6.47
9	200	40	0.75	103.07	37.86
10	250	40	1.00	45.84	18.45
11	300	60	0.75	94.97	45.30
12	250	50	0.75	64.54	24.26
13	200	50	0.50	26.69	19.16
14	200	60	0.75	52.68	27.52
15	300	50	0.50	10.17	13.82

Table 2. Analysis of variance of the regression parameters of the predicted response surface quadratic models.

Source of variation	Dependent variable									
	Kaempferitrin (KM)					Astragalins (KG)				
	DF	SS	MS	F-Value	p-Value	DF	SS	MS	F-Value	p-Value
Model	4	13949.1	3487.3	7.69	0.004	6	1537.13	256.19	9.40	0.003
Linear	3	10297.1	3432.4	7.57	0.006	3	572.80	190.93	7.01	0.013
A	1	2.1	2.1	0.00	0.948	1	13.99	13.99	0.51	0.494
B	1	0.0	0.0	0.00	0.999	1	84.73	84.73	3.11	0.116
C	1	10295.1	10295.1	22.72	0.001	1	474.08	474.08	17.40	0.003
Quadratic	1	3651.9	3651.9	8.06	0.018	2	644.95	322.48	11.83	0.004
A <sup>2</sup>	1	3651.9	3651.9	8.06	0.018	1	451.32	451.32	16.56	0.004
C <sup>2</sup>	-	-	-	-	-	1	152.82	152.82	5.61	0.045
Interactions	-	-	-	-	-	1	319.38	319.38	11.72	0.009
AB	-	-	-	-	-	1	319.38	319.38	11.72	0.009
Error	10	4532.2	453.2			8	218.02	27.25		
Lack of fit	8	3850.8	481.4	1.41	0.479	6	125.90	20.98	0.46	0.807
Pure error	2	681.4	340.7			2	92.12	46.06		
Total	14	18481.3				14	1755.15			
R <sup>2</sup>			0.7548					0.8758		
R <sup>2</sup> <sub>adj</sub>			0.6567					0.7826		
R <sup>2</sup> <sub>pred</sub>			0.4209					0.6118		
APV			4.88					7.53		
AAD			11.87					4.77		
PRESS			10702.52					681.33		

A = Pressure. B = Temperature. C = Co-solvent flow rate. DF = Degrees of freedom. SS = Sum of squares. MS = Mean square. R<sup>2</sup> = Coefficient of determination. R<sup>2</sup><sub>adj</sub> = Adjusted determination coefficient. R<sup>2</sup><sub>pred</sub> = Predicted determination coefficient. APV = Adequate precision value. AAD = Absolute average deviation. PRESS = Predicted residual error of sum of square.

Values of R<sup>2</sup> close to 1 and an AAD (absolute average deviation) as small as possible indicate that the model equation defines the true behavior of the system and it can be used for interpolation in the experimental domain [36, 39]. Moreover, the APV (adequate precision value) is an index of the signal to noise ratio and a value greater than 4 is an essential requirement for a model to be a good fit [37]. According to Myers et al. [38] if R<sup>2</sup> and R<sup>2</sup><sub>adj</sub> differ substantially, there is a strong possibility that nonsignificant terms have been included in the model. In

accordance with the results obtained for  $R^2$ , AAD (11.87 and 4.77) and APV (4.88 and 7.53), the equations of the models (2 and 3) satisfied the aforementioned thus the concentration of kaempferitrin and astragalín in muicle can be expressed in terms of the following regression equations:

$$KM = 718.0 - 6.27(A) - 0.001(B) + 143.5(C) + 0.0125(A^2) \quad (2)$$

$$KG = 428.4 - 3.125(A) - 4.14(B) + 184.7(C) + 0.0044(A^2) - 102.6(C^2) + 0.0179(AB) \quad (3)$$

### 3.3 Extraction process optimization

The regression models allowed predictions of the effects of independent variables on the kaempferitrin (KM) and astragalín (KG) yields from muicle (*J. spicigera*). The relationship between independent and dependent variables is shown in the three-dimensional representation of the response surfaces generated by the models (Fig. 3). A significant effect ( $p \leq 0.05$ ) between pressure and temperature (Fig. 3d) was observed on the KG yields, by increasing pressure and extraction temperature, highest concentrations are achieved. In the case of KM yields (Fig. 3a), no significant effect was observed. Regarding pressure and co-solvent flow rate (Fig. 3b and 3e), no significant effect on the KM and KG yields was observed. However, a positive correlation between pressure and co-solvent flow rate can be observed. The same has occurred with the temperature and co-solvent flow rate interaction (Fig. 3c and 3f). Furthermore, it can be observed that the KM and KG yields are the same at pressures of 200 and 300 bar at different co-solvent flow rates (Fig. 3b and 3e). The most influential factor from regression models (Eqs. 2 and 3) was the co-solvent flow rate.

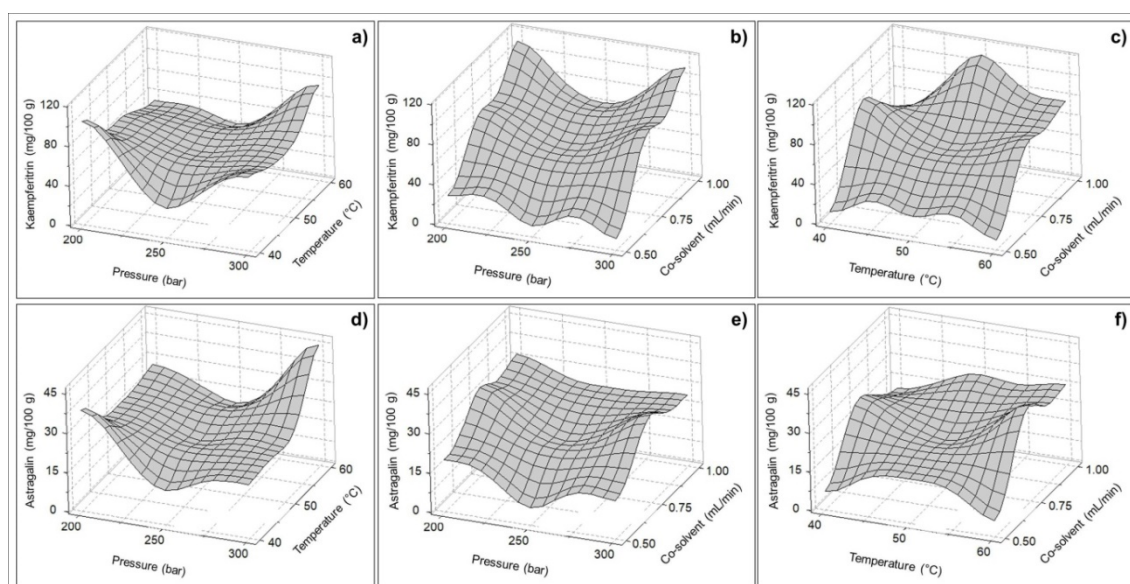


Figure 3. Response surface graphs for kaempferitrin and astragalín content (experimental data) as a function of pressure, temperature, and co-solvent flow rate.

In general, a similar behavior can be observed between KM and KG concentration with regard to independent variables. This was confirmed by obtaining a positive correlation (Fig. 4) between KM and KG concentrations, therefore the extraction conditions showed a similar effect in both compounds.

Overall, higher yields showed a tendency toward high pressures, high temperatures, and high co-solvent flow rates. The foregoing differs from that reported by Liza et al. [34] who concluded that the pressure had a higher influence on the yield in the extraction of *Strobilanthes crispus* leaves, using supercritical CO<sub>2</sub> and ethanol (99.5 %) as co-solvent. The best conditions were at pressure 200 bar, temperature 50° C and time 60 min, keeping the flow rates of CO<sub>2</sub> and ethanol constant (10 y 1 g/min, respectively). Eight flavonoids were identified in the extract obtained (3.98 % total flavonoids) with the optimal conditions, of which the highest concentration was kaempferol (19.45 mg/g). Similar results were obtained by Wang et al. [35] using supercritical CO<sub>2</sub> to obtain bioactive compounds from *Ampelopsis grossedentata* stems. Highest content of total flavonoids (4.67±0.36 mg routine equivalents/g dry sample) and total phenols (2.49±0.10 mg gallic acid equivalents/g dry sample) were obtained at 250 bar, 40° C, 50 min, with a ratio methanol:ethanol of 1:3 for total flavonoids and 1:1 for total phenols. Authors conclude that by decreasing supercritical CO<sub>2</sub> density, influences extraction more, than increasing vapor pressure of solute.

On the other hand, Li et al. [33] implemented the response surface methodology to obtain two kaempferol glycosides, KG1 (kaempferol-3-O-[2-O-β-D-galactopyranosyl-6-O-α-L-rhamnopyranosyl]-β-D-

glucopyranoside) and KG2 (kaempferol-3-O-[2-O- $\beta$ -D-xylopyranosyl-6-O- $\alpha$ -L-rhamnopyranosyl]- $\beta$ -D-glucopyranoside), from tea seed cake of *Camellia sinensis* (by-product produced by pressing the seeds of green tea during traditional processing to obtain oil), by supercritical fluid extraction. The optimal conditions were: extraction time 150 min, pressure 20 MPa (200 bar), temperature 80° C and 60 % ethanol. The flow rates of CO<sub>2</sub> and the co-solvent was maintained at 2 L/min and 0.5 mL/min, respectively. Authors conclude that higher yields were correlated with lower pressures and with the increase in temperature, probably due to the higher speed of mass transfer.

According to response surface analysis, the highest concentration of KM (109.0 mg/100 g dry powder) was achieved at 300 bar, 60° C, and a co-solvent flow rate of 1.0 mL/min. In the case of KG, the highest concentration (44.07 mg/100 g of dry powder) was achieved at 300 bar, 60° C, and a co-solvent flow rate of 0.9 mL/min. Under these conditions (300 bar, 60° C, and a co-solvent flow rate of 1.0 mL/min, with an extraction time of 180 min and a CO<sub>2</sub> flow rate of 5 mL/min), extractions were performed (in triplicate), and concentrations obtained for KM and KG were 115.08 $\pm$ 2.81 and 56.63 $\pm$ 9.02 mg/100 g of dry powder, respectively. Comparing the calculated values with the experimental ones by using a t-test, there were no significant differences, therefore the quadratic models are suitable.

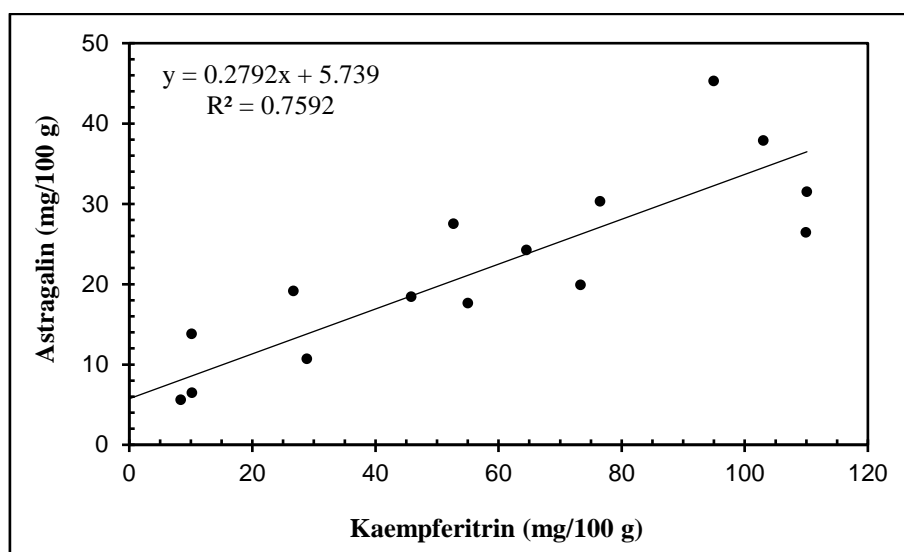


Figure 4. The Relationship between kaempferitrin and astragaline concentration in muicle (*J. spicigera*) extracts obtained by supercritical fluids.

### 3.4 Comparison of extraction methods

The effect of temperature and extraction time on the KM and KG yields in muicle extracts obtained by the conventional method are shown in Table 3. It can be observed that the higher the temperature and the higher the extraction time, the higher the yields of KM and KG. Treatment 4 showed the highest yields of KM and KG (574.20 $\pm$ 65.10 and 113.10 $\pm$ 15.06 mg/100 g of dry powder, respectively). These results are higher than those obtained by supercritical extraction under optimal conditions (115.08 $\pm$ 2.81 and 56.63 $\pm$ 9.02 mg/100 g of dry powder, respectively), equivalent to 20 and 50 % of KM and KG, respectively; compared to the yields obtained in conventional extraction for treatment 4.

Table 3. Effect of temperature and extraction time on kaempferitrin (KM) and astragaline (KG) yields in muicle extracts obtained by conventional extraction.

Treatment	Temperature (°C)	Time (min)	KM (mg/100 g)	KG (mg/100 g)
1	50	60	348.20 $\pm$ 25.90 a	59.74 $\pm$ 7.50 a
2	50	120	461.18 $\pm$ 5.02 b	80.24 $\pm$ 9.15 a
3	70	60	477.60 $\pm$ 36.82 bc	61.20 $\pm$ 8.84 a
4	70	120	574.20 $\pm$ 65.10 c	113.85 $\pm$ 15.20 b

Values represent the mean  $\pm$  standard deviation of three observations. Values bearing different letters in the same column are significantly different ( $p \leq 0.05$ ).

Because these flavonols contain sugar molecules in their structure, this makes them more soluble in aqueous solutions of ethanol, than in pure ethanol, due to the hydroxyl groups [29, 40]. Therefore, new extractions (in

triplicate) were performed, using 70 % (by mass) ethanol as co-solvent and according to the optimal conditions achieved in the response surface analysis (300 bar, 60 °C, and a co-solvent flow rate of 1.0 mL/min). The results showed a higher concentration of KM and KG ( $562.71 \pm 156.85$  and  $79.90 \pm 18.03$  mg/100 g of dry powder, respectively) compared to the 99.5 % ethanol extractions. The use of 70 % ethanol as co-solvent in the supercritical extraction process considerably improved the yields of KM and KG (98.0 and 70.0 %, respectively, compared to the yields obtained in conventional extraction for treatment 4). Figure 5 shows the chromatograms obtained for the standards (kaempferitrin and astragalín), the muicle extracts (*J. spicigera*) obtained by supercritical fluids, and the conventional extraction.

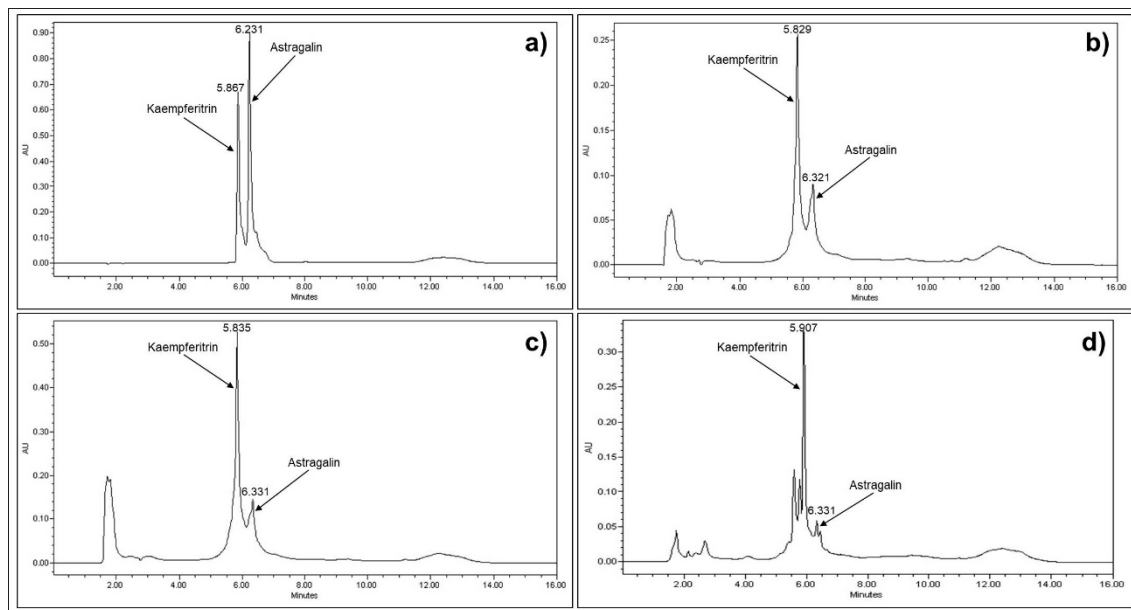


Figure 5. Chromatograms obtained by HPLC at 265 nm. **a)** Standards solution. **b)** Muicle extract obtained with the optimal conditions by supercritical fluids (99.5 % ethanol as co-solvent). **c)** Muicle extract obtained with the optimal conditions by supercritical fluids (70 % ethanol as co-solvent). **d)** Muicle extract obtained by conventional extraction (70° C and 120 min).

#### 4. Conclusions

Adequate fits were achieved and most of the variability of the responses was explained by the models obtained. Supercritical fluid extraction of KM and KG from muicle (*J. spicigera*), was optimized at 300 bar, 60° C, and 1.0 mL/min of co-solvent flow rate, with a constant CO<sub>2</sub> flow rate of 5 mL/min and extraction time of 180 min. In accordance with the KM and KG concentration, the most influential variable was the co-solvent flow rate. Moreover, a positive correlation could be observed between KM and KG concentration, suggesting that the extraction conditions showed a similar effect in both compounds. The use of 70 % ethanol as co-solvent significantly improved the yields of KM and KG, compared to pure ethanol. Adequate yields were achieved of KM and KG by supercritical fluid extraction compared with conventional extraction (98 and 70 %, respectively). However, it is recommended to perform extractions at different concentrations of ethanol aqueous solutions as co-solvent, with the aim of increasing KM and KG yields. Taking into account the functional properties of these flavonols (kaempferitrin and astragalín), *J. spicigera* extracts obtained by supercritical fluids could be used in the development of functional foods, as well as its possible use in traditional medicine by the health professionals.

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