*Bulgarian Chemical Communications, Volume 51, Issue 1, (pp. 80 – 87) 2019*

# Development and validation of a chromatographic method to identify and quantify the flavonoids extracted from *S. rebaudiana* Bertoni

A. Carrera-Lanestosa[1], M. Segura-Campos[1], T. Coral-Martinez[2], D. Ruíz-Ciau[2], J. Araujo-León[2], D. Betancur-Ancona[1], Y. Moguel-Ordóñez[3*]

[1]*Facultad de Ingeniería Química, Universidad Autónoma de Yucatán, Mérida, México (Posgrado Institucional en Ciencias Químicas y Bioquímicas)*
[2]*Facultad de Química, Universidad Autónoma de Yucatán, Mérida, México*
[3]*Campo Experimental Mocochá, CIRSE-INIFAP, Mocochá, México*

Received August 8, 2018; Revised September 5, 2018

Leaf extracts of *Stevia rebaudiana* contain a great variety of flavonoids which are potential antioxidants for the human body. In this work a high-performance liquid chromatography (HPLC)-based method to identify and quantify flavonoids (quercetin, kaempferol, luteolin and apigenin) from extracts of *S. rebaudiana* was developed and validated. A HPLC with a diode detector array at 200 nm wavelength and an Eclipse XDB-C18 column (150 mm length, 4.6 mm d.i., and 5 μm particle size) was used. The mobile phase was acidified methanol (pH 2.5) in a gradient elution system. Sample injection volume was 10 μL at 1 mL/min flow rate and 25 °C temperature. Aqueous extracts of *S. rebaudiana* were prepared and its flavonoids quantified with the developed method. The validation of the method was performed according to the ICH Q2A guideline, that is, assessing selectivity, linearity, sensitivity, precision, detection limits (DL), quantification limits (QL), and accuracy. Calibration curves showed a linear trend with a correlation and determination coefficient higher than 0.99. Precision showed relative standard deviation (RSD) values lower than 10%. DLs and QLs were 1.93 and 3.35 – 6.43 μg/mL, respectively, while accuracy showed 90 – 100% recovery of fortified samples. The implemented method fulfills the metrics of the ICH and is suitable to identify and quantify flavonoids in *S. rebaudiana* Bertoni.

**Keywords:** *Stevia rebaudiana*, Flavonoids, ICH, Validation, HPLC.

## INTRODUCTION

Several pharmacological, clinical, toxicological and therapeutic applications of leaf extracts of *S. rebaudiana* [1, 2], as well as numerous biological activities such as antacid, anticaries [3], anti-rotavirus, antibacterial [4], antihypertensive, antifungal, anti-inflammatory, antiviral, anti-yeast, diuretic, antioxidant, anti-cancer, etc., have been found [5].

*S. rebaudiana* is currently grown commercially for use as a sweetener as it contains diterpene glycosides [6], stevioside and rebaudioside A being most common in the leaves (5 – 10% and 2 – 4% w/w on a dry basis, respectively). On the other hand, rebaudioside B, rebaudioside C, rebaudioside D, rebaudioside E, rebaudioside F, dulcoside A, rubusoside, and esteviolbioside are less common glycosides in *S. rebaudiana* leaves [7].

*S. rebaudiana* leaves also contain a complex mixture of other compounds, such as diterpenes, labdanos, triterpenes, stigmasterol, tannins, ascorbic acid, alkaloids, steroids, saponins, β-carotene, chromium, cobalt, magnesium, iron, potassium, phosphorus, riboflavin, thiamin, tin, zinc, β-sitosterol, caffeic acid, campesterol, caryophyllene, centaureidin, chlorogenic acid, chlorophyll, flavonoids, among others [8]. Flavonoids are bioactive compounds present in fruits, vegetables, grains, nuts, seeds, spices, medicinal plants, and some usual drinks such as tea, red wine, and beer [9]. Flavonoids are of scientific interest due to their antioxidant activity which makes them suitable for stabilizing and eliminating oxygen reactive species (ORS). Furthermore, flavonoids have other beneficial effects including anti-inflammatory, antimicrobial and antidiabetic, antihypertensive, and anticancer effects [8, 9]. Flavonoids have a base structure consisting of two benzene rings at the ends of the molecule joined by a 3-carbon ring. Other molecules, such as methyl, oxyhydrides, and sugars, can be added to the 3-carbon ring, making possible the generation of different types of flavonoids such as flavanones, flavonols, catechins, flavones, anthocyanins, and isoflavones [10].

Some flavonoids, such as quercetin, kaempferol, luteolin and apigenin (Fig. 1), are present in *S. rebaudiana*. However, the content of these compounds highly depends on the stress of the plant as a consequence of the type of soil, weather, agronomic conditions, or disease [8, 9].

* To whom all correspondence should be sent:
E-mail: moguel.yolanda@inifap.gob.mx

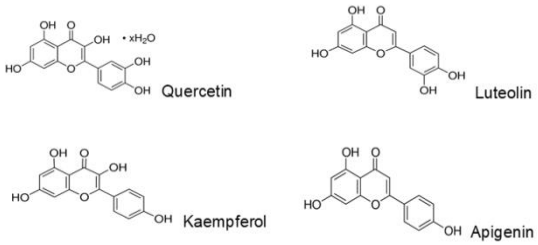


**Fig 1.** Chemical structure of the flavonoids in *S. rebaudiana* Bertoni [9].

There are several methods to identify and quantify flavonoids in *S. rebaudiana* including colorimetric [8, 11, 12] and spectrophotometric methods, [4, 13, 14], thin-layer chromatography (TLC) [9], high-performance liquid chromatography (HPLC) [15], and others. HPLC is frequently used to identify and quantify diverse compounds in numerous biological extracts with a high precision and reliability. Currently, there is no official method to identify and quantify flavonoids in *S. rebaudiana*. Therefore, this works aims to develop and validate a HPLC-based method to identify and quantify the flavonoids (quercetin, kaempferol, luteolin, and apigenin) from aqueous leaf extracts of *S. rebaudiana* Bertoni.

## EXPERIMENTAL

### *Reactive and standard reference materials*

Two standard reference materials were used for flavonols and flavones: the one contained quercetin (Sigma PHR1488) and kaempferol (Sigma 96353), and the second contained luteolin (Sigma 72511) and apigenin (Sigma 42251), respectively. Standard flavonoids were diluted with deionized water (Milli Q water) to prepare the mother solutions (1 mg/mL) used to generate the required calibration curves to validate the method. These solutions were filtered through a nylon membrane (0.45 μm) before injecting in the equipment.

### *Reactive and standard reference materials*

An Agilent Technologies equipment (model: 1200 series) with a diode detector array at 200 nm wavelength, packed with an autosampler, degasser, and temperature controller was used. Chromatographic separations were performed with a packed Eclipse XDB-C18 column (150 mm length, 4.6 internal diameter, and 5 μm particle size) (Agilent Technologies) at 25°C temperature. The mobile phase was methanol (HPLC-grade, J.T. Baker Phillipsburg, NJ) and acidified water (pH 2.5) using a system of gradient elution of 30 min, starting and ending with a water-to-methanol ratio of 3/7 and 7/3, respectively. The injection volume was 10 μL during 30 min (1 mL/min) for both, the standard reference materials and the samples.

### *Validating the method*

The developed method was validated under the metrics of the International Conference of Harmonization (ICH Q2A), which establishes the analytical parameters that must be assessed to validate a chromatographic method. The reliability of the method was determined with the parameters described below [16].

*Selectivity:* Selectivity refers to the capacity of the method to unequivocally assess the analyte in the presence of other components. All the standard reference materials were analyzed at 50 μg/mL according to the chromatographic conditions described earlier. The chromatographic peaks of each standard reference material were obtained, as well as their retention times ($t_R$) and ultraviolet-visible spectra.

*Linearity and sensitivity:* Linearity is the capacity of the method to obtain directly proportional responses to the content of analyte in the sample within a range. This parameter was assessed using the mother solutions under concentrations of 1 mg/mL for each flavonoid, which were diluted until reaching concentrations of 25, 50, 75, 100, 150, and 200 μg/mL to generate the calibration curves. The obtained contents were injected in triplicate and linearity was assessed by calculating the regression equation, as well as the correlation (r) and determination ($R^2$) coefficients. Sensibility was assessed as the slope of the equations of each analyte.

*Precision:* Intraday and interday precisions were assessed. The former was demonstrated by means of the analysis of a homogeneous sample under the same conditions and on the same day, obtaining a minimum grade of dispersion among the results. The second was performed by analyzing the sample under the same conditions but on different days. Both, intraday and interday precisions, must reach a relative standard deviation (RSD%) lower than 10% to consider the method precise. Intraday and interday RSD%, retention times ($t_R$), and areas under the curves were calculated considering the standard deviation (SD) and mean using equation 1.

$$\% RSD = SD/X \times 100 \quad (1)$$

*Detection and quantification limits (DL and QL):* DL is the lowest concentration of an analyte in a sample that can be detected but not necessarily quantified as an exact value. On the other hand, QL is the lowest concentration of an analyte contained in a sample that can be quantitatively determined

with an acceptable precision and accuracy. These factors were computed using equation 2.

$$DL = 3.3/SD \text{ and } QL = 10/SD, \quad (2)$$

3.3 and 10 being the standard deviation of the intercepts.

*Accuracy*: Accuracy refers to how close an identified value is to a predetermined value. The accuracy of the method was assessed by testing the recovery of analytes intentionally added to the aqueous extract of *S. rebaudiana* to fortify them. 100 µg/mL of each flavonoid was added to the aqueous extract of *S. rebaudiana* and each test was performed in triplicate. The recovery of analytes must range between 90% to 110%.

### *Aqueous extract of S. rebaudiana*

Aqueous leaf extracts of *S. rebaudiana* Bertoni from Yucatan, Mexico, were obtained by the method developed by Dutta *et al.* [17]. 10 g of milled leaves were suspended in 100 mL of distilled water (10% w/v). Extraction was performed in a 250 mL beaker in a Maria bath at 40 °C temperature under constant agitation during 3 h. Extracts were then centrifugated at 2500 rpm, 10 °C temperature for 30 min, and supernatants were recovered. Extraction was performed twice discarding the residue after the last washing. Supernatants were mixed and filtered at vacuum using Whatman 2 filter papers, and were lyophilized using a Labconco FreeZone 4.5 lyophilizer.

### *Identification and quantification of flavonoids in the aqueous extract of S. rebaudiana Bertoni*

After validating the chromatographic method, flavonoids from *S. rebaudiana* were identified by superposition of the standard reference materials with the extract. As the vegetable material has a complex chemical composition, as well as a high portion of analytes and interferent materials, the UV-visible spectral region of the peaks identified in the extract was used as comparison criterion to confirm or highlight the presence of analytes. A 10-mg aliquot of lyophilized extract was diluted in 3 mL of Milli Q water, collected in a 5 mL volumetric flask, filtered through a 0.45-µm nylon membrane, and analyzed.

### *Statistical analysis*

Regression analysis was performed to assess the linearity by the least-square method using the Statgraphics CENTURION XV software. Microsoft Excel spreadsheets were configured to analyze the precision, DL, QL, and accuracy parameters.

## RESULTS AND DISCUSSION

### *Validating the method*

*Selectivity:* Chromatographic peaks were obtained during the analysis of the flavonoids and their $t_R$ were identified. The presence of the analytes was verified with their UV-visible spectra to check the selectivity of the method (Fig 2).

The $t_R$ of the assessed analytes were shorter than those reported by Muanda *et al*. [18], who got $t_R$ for quercetin and apigenin of 42.87 and 44.89 min, respectively, using a mobile phase (50 mM ammonium phosphate, 80% acetonitrile, and 200 mM phosphoric acid) and a C18 intersil ODS column. Toso & Skliar, [19], got a $t_R$ of 33.12 min for kaempferol using a mobile phase in methanol-water (15:85 – 100:0) gradient and a RP-C18 column, while Xiao-quing & Jian-bo, [20] reported a $t_R$ of 20.1 min for luteolin using a mobile phase consisting of a mixture of methanol, acetonitrile, acetic acid, and phosphoric acid (200:100:10:10:200 v/v) and a Kromasil RP-C18 column. The polarity among the analytes and the mobile phase, along with the type of column, might explain the differences in $t_R$s. A C-18 column, which can retain each component depending on its affinity, was used in all the cases (octadecyl functional group). However, this was not the case for the mobile phases which play a relevant role on $t_R$s. Flavonoids are non-polar analytes and have affinity for less polar mobile phases. Muanda *et al.* [18] and Xiao-quing & Jian-bo, [20] used salts, acids, and/or acetonitrile as mobile phases, which are more polar than methanol. In this sense, the assessed analytes were retained much longer in a chromatographic column due to the affinity and difference of polarities. Toso & Skliar, [19] used a gradient mobile phase similar to that of this study but using a methanol-water gradient of (15:85) at the beginning. In minute 32 the less polar solvent reached a higher percentage (100:0) and therefore, kaempferol was eluted from the column in 33.12 min $t_R$, which is longer than in this study using gradient elution (water-methanol 70:30 v/v). However, since the beginning the percentage of methanol increased quickly, which allowed for eluting the analytes in a shorter time. It is important to highlight that the $t_R$ obtained in this study under the developed method have a great advantage as they are shorter and require a lower volume of the mobile phase to determine the flavonoids from *S. rebaudiana*.

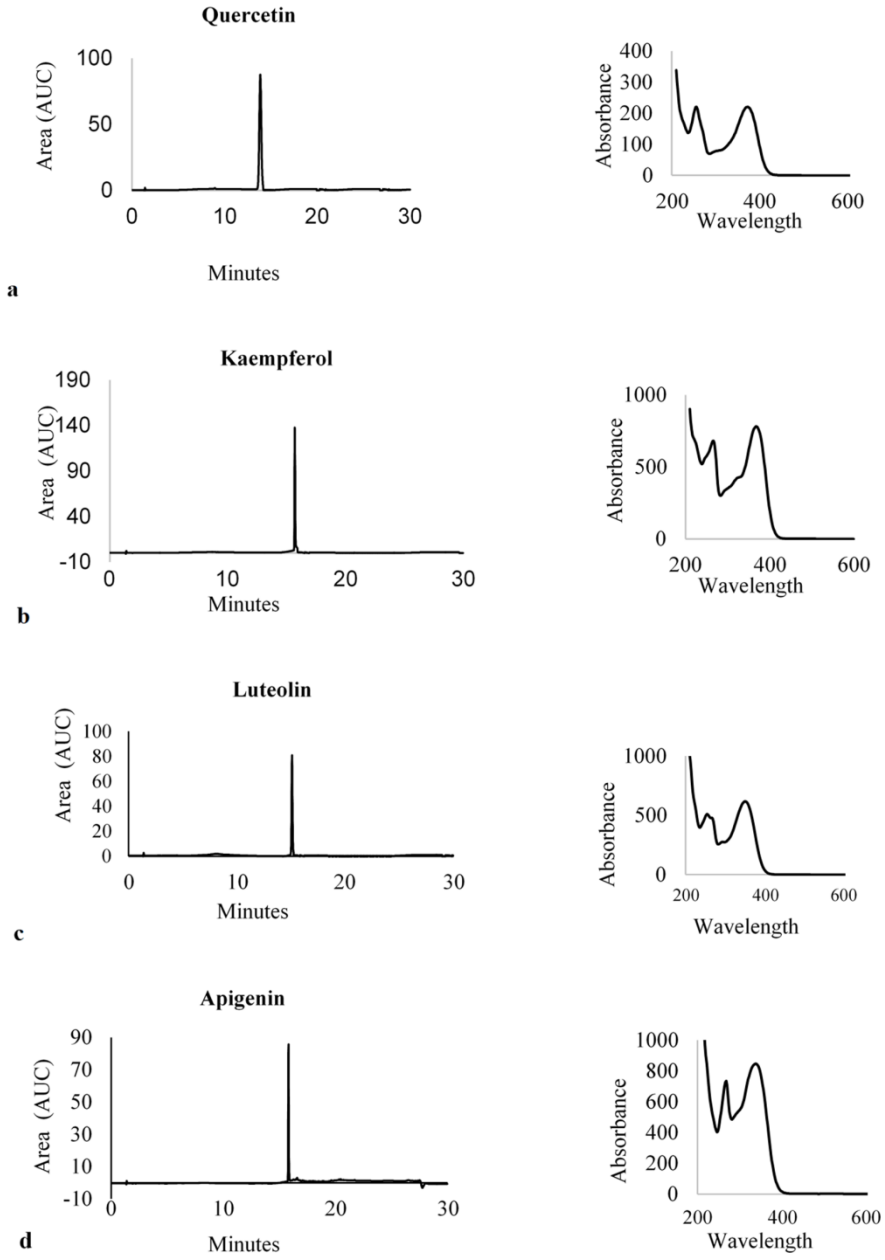


**Fig. 2.** Retention times ($t_R$) and UV spectra of the flavonoids at a concentration of 50 µg/mL. a) quercetin ($t_R$ 13.88 min), b) kaempferol ($t_R$ 15.62 min), c) luteolin ($t_R$ 15.08 min), d) apigenin ($t_R$ 15.81 min).

*Linearity and sensitivity:* The linearity of the developed method was appropriate to quantify flavonoids as it showed a linear regression between the concentration of the standard reference material and the area under the curve of the chromatographic analysis. The correlation coefficient (r) showed a linear trend between the two of the analyzed variables (area under the concentration curve) of 0.99. The determination coefficient ($R^2$) was higher than 99% in most of the cases but kaempferol, for which it was 98%. The intercepts refer to the point where two lines meet, while the values of the slopes - to the inclination of the line in the linear model (Fig 3). Luteolin slopes had a higher inclination (23.48) than apigenin (16.73), which indicates a greater sensitivity to the developed method than the slopes with a lower inclination, such as those for quercetin and kaempferol (12.69 and 12.60, respectively) (Table 1).

**Table 1.** Parameters of the linear regression model (y = a + b*x) of the standard reference materials of flavonoids (quercetin, kaempferol, luteolin, and apigenin).

| Parameter | Quercetin | Kaempferol | Luteolin | Apigenin |
|---|---|---|---|---|
| r | 0.999 | 0.993 | 0.999 | 0.998 |
| $R^2$ | 0.999 | 0.987 | 0.999 | 0.997 |
| Intercept in the Y axis | -50.52 | 732.95 | 147.81 | 989.66 |
| Slope | 12.69 | 12.60 | 23.48 | 16.73 |

R: correlation coefficient, $R^2$: determination coefficient.

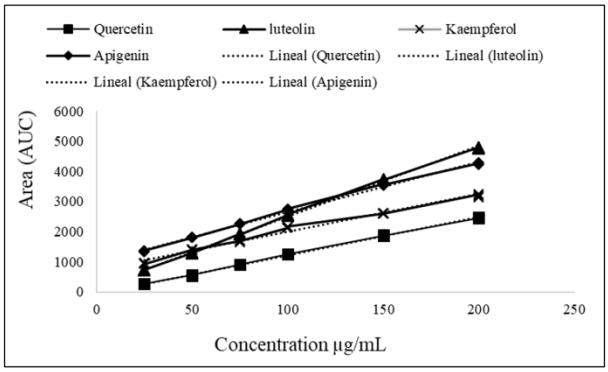


**Fig. 3.** Linear regression graphic (mean ± SD). Area (AUC) *vs* concentration (y = a + bx) for quercetin, kaempferol, luteolin and apigenin.

*Precision:* Concentrations of 50, 100, and 200 µg/mL were used to measure the intraday precision. As indicated in Table 2, the RSD% for $t_R$ was lower for luteolin (0.04%), and higher for quercetin (0.22%) and kaempferol (1.55%). The concentrations used to measure interday precision were the same. In this case, the RSD% for $t_R$ was lower for kaemperfol (0.01%) and higher for quercetin (0.45%). The RSD% values of the area under the curve were lower than 9%, obtaining a lower value for apigenin (0.56%) and a higher value for kaempferol (8.68%).

**Table 2.** Intraday precision of the standard reference materials of flavonoids.

| Flavonoid | Conc. (µg/mL) | $t_R$ (Mean ± SD) | RSD% Time | Area (Mean ± SD) | RSD% Area |
|---|---|---|---|---|---|
| Quercetin | 50 | 13.86±0.03 | 0.22 | 547.63±6.57 | 1.20 |
| | 100 | 13.84±0.01 | 0.08 | 1247.37±2.74 | 0.22 |
| | 200 | 13.80±0.01 | 0.07 | 2469.9±16.11 | 0.65 |
| Kaempferol | 50 | 15.62±0.02 | 0.09 | 1388.47±9.8 | 0.71 |
| | 100 | 15.61±0.005 | 0.04 | 2149.27±24.3 | 1.13 |
| | 200 | 15.60±0.005 | 0.04 | 3191.6±49.5 | 1.55 |
| Luteolin | 50 | 15.07± 0.01 | 0.04 | 1282.13±10.7 | 0.84 |
| | 100 | 15.07±0.01 | 0.04 | 2546.5±16.2 | 0.64 |
| | 200 | 15.07±0.01 | 0.04 | 4788.47±15.6 | 0.33 |
| Apigenin | 50 | 15.81±0.01 | 0.04 | 1804.07±6.70 | 0.37 |
| | 100 | 15.80±0.005 | 0.06 | 2739.43±6.48 | 0.24 |
| | 200 | 15.80±0.005 | 0.04 | 4257.07±34.9 | 0.82 |

**Table 3.** Interday precision of the standard reference materials of flavonoids.

| Flavonoid | Conc. (µg/mL) | $t_R$ (Mean ± SD) | RSD% Time | Area (Mean ± SD) | RSD% Area |
|---|---|---|---|---|---|
| Quercetin | 50 | 13.88±0.06 | 0.22 | 545.48±6.4 | 1.20 |
| | 100 | 13.85±0.04 | 0.08 | 1243.49±7.0 | 0.22 |
| | 200 | 13.83±0.05 | 0.07 | 2468.63±15.6 | 0.65 |
| Kaempferol | 50 | 15.62±0.01 | 0.09 | 1416.74±38.8 | 0.71 |
| | 100 | 15.61±0.007 | 0.04 | 2072.27±179.8 | 1.13 |
| | 200 | 15.55±0.17 | 0.04 | 3116.11±136.8 | 1.55 |
| Luteolin | 50 | 15.07±0.02 | 0.04 | 1254.13±22.6 | 0.84 |
| | 100 | 15.07±0.01 | 0.04 | 2525.08±22.5 | 0.64 |
| | 200 | 15.07±0.01 | 0.04 | 4758.32±28.8 | 0.33 |
| Apigenin | 50 | 15.81±0.007 | 0.04 | 1812.38±20.5 | 0.37 |
| | 100 | 15.80±0.007 | 0.06 | 2742.77±12.2 | 0.24 |
| | 200 | 15.81±0.007 | 0.04 | 4260.12±28.6 | 0.82 |

In both cases the RSD% was lower than 10%, indicating a minimum grade of dispersion in the results (Table 3). This demonstrates that the method is precise as it is under the predetermined acceptance criteria.

**Table 4.** Detection limits (DL) and quantification limits (QL) of the standard reference materials of flavonoids.

| Flavonoid | Detection Limit (DL) (µg/mL) | Quantification Limit (QL) (µg/mL) |
|---|---|---|
| Quercetin | 1.00 | 3.35 |
| Kaempferol | 1.76 | 5.85 |
| Luteolin | 1.93 | 6.46 |
| Apigenin | 1.19 | 3.99 |

*Detection and quantification limits:* The DL of the flavonoids ranged between 1.00 and 1.93 µg/mL, quercetin and luteolin being detected at 1.00 and 1.93 µg/mL, respectively. The concentrations of flavonoids that could be quantified with an acceptable precision (QL) ranged between 3.35 and 6.46 µg/mL. In this case, quercetin displayed a lower value of the quantification limit (3.35 µg/mL), while for luteolin the concentration was higher (6.46 µg/mL). Quercetin can be detected and quantified under lower concentration as it contains a higher number of fluorescent groups which provide a higher molecular sensitivity (Table 4).

*Accuracy:* Accuracy was measured using the extract of *S. rebaudiana* fortified with the 4 flavonoids recovering 94.28 – 100.86% of the added analytes (Table 5), luteolin being the most recovered (100.86%) analyte, followed by quercetin (100.73%). On the other hand, kaempferol and apigenin were the least recovered analytes (94.28 and 96.70%, respectively). A higher content of luteolin and quercetin in the extract of S. *rebaudiana* explains these differences. These results indicate that the developed method is accurate and does not lead to losses in the assessed analytes as it is within the predetermined acceptable criteria.

**Table 5.** Accuracy of the analytical method expressed as the recovery percentage of the samples fortified with the standard reference materials of flavonoids

| Flavonoid | Accuracy (%) Mean ± SD |
|---|---|
| Quercetin | 100.73±1.10 |
| Kaempferol | 94.28±2.08 |
| Luteolin | 100.86±0.58 |
| Apigenin | 96.70±0.64 |

Samples were fortified with 100 µg/mL of each standard reference material.

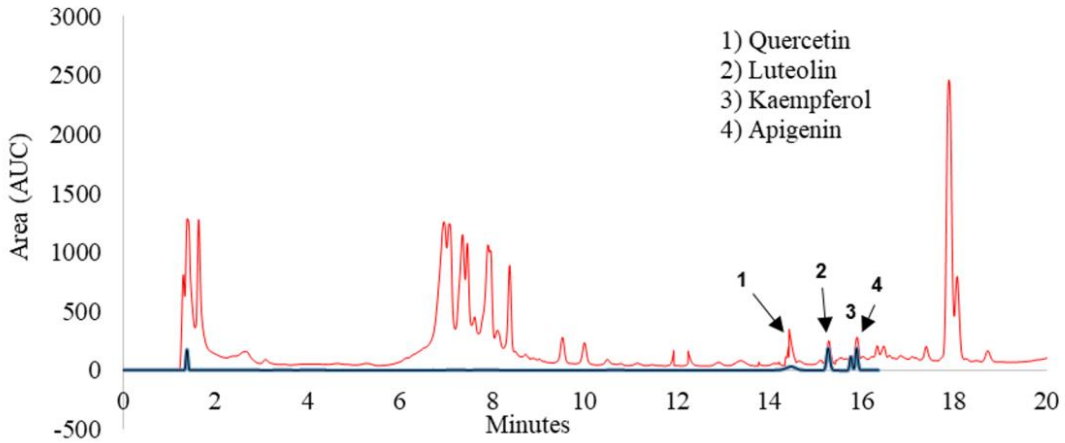


**Fig. 4.** Chromatogram of the extract of *S. rebaudiana* Bertoni.

*Identification and quantification of the flavonoids from the aqueous extracts of S. rebaudiana Bertoni*

Fig. 4 shows the chromatographic analysis to identify and quantify the flavonoids from the extract of *S. rebaudiana*. Both the chromatographic peaks and the superposition of the 4 flavonoids have the same $t_R$.

Table 6 shows the quantification of the flavonoids through the calibration curves performed during the validation process. Quercetin, luteolin, and apigenin contents identified in this work were higher than those reported by Periche *et al.* (2016), who assessed the hydro alcoholic extracts of *S. rebaudiana* leaves by HPLC and found a high

content of quercetin and apigenin of 0.039 g/100g of dried leaves for both analytes. This may be due to the fact that the presently analysed sample of *S. rebaudiana* was from a new genetic material that has been selected considering its agronomic conditions which were adapted to the conditions in the southeastern zone of Mexico. Thus, *S. rebaudiana* grew under pretty particular conditions including a leptosol soil (not very deep soils, dark color limited by rock, retains up to 80% water, and has a high salt content which means high electric conductivity). This created stressful conditions for Stevia, which could cause a higher phytochemical content.

**Table 6.** Flavonoid content expressed as g/100g of leaves of *S. rebaudiana*.

| Flavonoid | *S. rebaudiana* Mean ± SD |
|---|---|
| Quercetin | $0.062 \pm 0.5^{b}$ |
| Kaempferol | ND[a] |
| Luteolin | $0.084 \pm 0.2^{d}$ |
| Apigenin | $0.079 \pm 0.06^{c}$ |

ND: not detected. [a-d] Different letters indicate statistical difference ($P < 0.05$).

Regarding the luteolin and kaempferol contents in *S. rebaudiana*, no reports about their quantification by HPLC were found. Mehenni *et al.* [21] found luteolin (0.061g/100 g dried leaves) in ethanolic extracts of *P. lentiscus* leaves. Ghanta *et al.* [9] characterized kaempferol-3-O-ramnoside in a derivate of kaempferol containing a rest of rhamnose in the C-3 position of the original compound. The presence of kaempferol was not detected in the assessed extracts possibly because its content is below the detection and quantification limits reported in the validation stage. Furthermore, the kaempferol used in this study was the original compound and not the characterized glycosylate used by Ghanta *et al.* [9].

López-Corona [22] reported an 8.64 µg/mL content of kaempferol in the hydrolyzed methanolic extract of *C. graciliar* leaves using low concentrations in the standard curve. The extract could be detected and therefore, the differences with this work might rely on the type of vegetable species used, as well as on the geo-climatic and agronomic conditions of the crop.

## CONCLUSIONS

The developed method was validated and was found selective, linear, sensitive, precise, and accurate within the ranges predetermined by the ICH. Therefore, this method can be applied to identify and quantify flavonoids extracted from *S. rebaudiana* Bertoni cultivated in southeastern Mexico.

***Acknowledgement:*** *This work was supported by the Fiscal Funds Program-INIFAP through the "Development of based food products based on Stevia rebaudiana Bertoni leaves" project. We also want to thank to the Program for Improvement of Professor [Programa de Mejoramiento al Profesorado (PROMEP)] by the given scholarship.*

## REFERENCES

1. K. Das, R. Dang, N. Gupta, *Int. J. Nat. Eng. Sci.*, **3**, 65 (2009).
2. D. Preethi, T.M. Sridhar, P. Josthna, C.V. Naidu,. *J. Ecobiotech.*, **3**, 05 (2011).
3. S. A. González-Moralejo, An approach to understand a potential natural sweetener, *S. rebaudiana* Bertoni: production, consumption, and potential demand. [In Spanish]. *Agro.*, **17**, 57 (2011).
4. S. Singh, V. Garg, D. Yadav, M. N. Beg, N. Sharma, In vitro antioxidative and antibacterial activities of various parts of S. rebaudiana (Bertoni). *Intern. J Pharmacy Pharmaceutical Sci..*, **4**, 468 (2012).
5. J. R. Ramirez, O. Y. Moguel, *Stevia rebaudiana* Bertoni, a crop with a productive, sweetener and medicinal potential in México, in: D. Betancur-Ancona (ed.) *S. rebaudiana*, Chemical composition, uses and health promoting aspects, Yucatán, México: Nova, 2015, p. 1.
6. K. Y. Ashok, D. Singh, P. S. Ahuj, *Can. J. Plant Sci.*, **91**, 1 (2011).
7. M. C. Carakostas, A. C. Curry, D. J. Brusick, *Food Chem. Toxicol.*, **46**, S1 (2008).
8. S. Bakkar, M. M. R. Rahman, M. A. Hossain, M. A. Rashid, *J. Trop. Asian Pac. Dis.*, **4**, 275 (2014).
9. S. Ghanta, A. P. Banerjee, S. Chattopadhyay, *J. Agric. Food Chem.*, **55**, 10962 (2007).
10. J. C. I. Escamilla, M. E. Y. Cuevas, F. J. Guevara, *J. Rev. Fac. Med.*, **52**, 73 (2009).
11. K. Il-Suk, Y. Mira, L. Ok-Hwan, *Food Science Technology*, **44**, 1328 (2011).
12. S. Sutradhar, U. Preeti, A. Humera, A. M. Muneem, *Res. J. Pharm, Biol. Chem. Sci.*, **14**, 674 (2013).
13. A. E. Abou-Arab, A. A. Abou-Arab, M. F. Abu-Salem., *Afric. J. Food Sci.*, **4**, 269 (2010).
14. E. Zayova, I. Stancheva, M. Geneva, M. Petrova, L. Dimitrova, *Turk J. Biol.*, **37**, 106 (2013).
15. A. Periche, M. L. Castelló, A. Heredia, I. Escriche, Effect of different drying methods on the phenolic, *J. Flavour Fragr.*, **31**, 173 (2016).
16. ICH. Harmonized tripartite guideline validation on analytical procedures: text and methodology. International Conference on Harmonization Q2A(R1) (2005).
17. P. K. Dutta, M. M. Razu, M. K. Alam, M. A. Awal, M. Mostofa, *Int. J. of Biol. Res.*, **2**, 17 (2010).

18. F. N. Muanda, R. Soulimani, B. Diop, A. Dicko, *Food Sci. Technol.*, **44**, 1865 (2011).
19. R. E. Toso, M. I. Skliar, Aislamiento, identificación y cuantificación de compuestos con actividad gastroprotectora presentes en Centaurea solstitialis. Ciencia Veterinaria. Facultad de Ciencias Veterinaria sp U.N.L. Pam, **17** (2002).
20. C. Xiao-qing, X. Jian-bo, RP-HPLC-DAD. *Iranian J. Pharm. Res.*, **3**, 175 (2005).
21. C. Mehenni, D. Kilani-Atmani, S. Dumarcay, D. Perrin, P. Gerardin, D. Atmani, *J. Food Drug Analysis*, **1** (2016).
22. M. G. López-Corona, Identificación y cuantificación del ácido clorogénico y de flavonoides presentes en hojas y frutos de *Crataegus gracilior* PHIPPS. Tesis de Licenciatura. Universidad Autónoma de Querétaro, Facultad de Química, 2013.