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Development of a quantitative high performance thin layer chromatographic method for analysis of caffeic acid and quercetin from cranberry extract

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GEORGIANA CRETU[1], EUGENIA EFTIMIE TOTU[1], ALEXANDRA RALUCA MIRON[1], AURELIA CRISTINA NECHIFOR[1]*
[1]University Politehnica of Bucharest, Analitycal Chemistry and Environmental Engineering Department, str. Polizu, nr.1-7, 011061, Bucharest, Romania
*Corresponding author e-mail: *nechiforus @yahoo.com*

Abstract

The paper presents a rapid and precise HPTLC method for quercetin and caffeic acid determination from cranberry extracst (Vaccinium macrocarpon). Quercetin and caffeic acid were extracted with a mixture of methanol and water and separated on HPTLC 60F_{254} silica gel plates with n-hexane, ethyl acetate and acetic acid 5:3:1 (v/v/v) as mobile phase within 15 minutes. In the second branch of the developing chamber was put hydrochloric acid 37 %. Densitometric analysis of these compounds was performed by fluorescence, using UV light at λ = 366 nm. The values obtained for R_f in the case of quercetin and caffeic acid respectively are 0.33 and 0.44. Calibration data revealed good linear relationship (R = 0.99984 and 0.99972) between peak height and quercetin and caffeic acid concentration. Based on the results obtained in this study we concluded that this method can be used for rapid routine analysis.

Keywords*:* HPTLC, cranberry, quantification, caffeic acid, quercetin, phenolic compounds, flavones

Introduction

The interest for natural antioxidants present in red fruits significantly increased in the last period. Berries are a good source of phenolic acids (caffeic acid), flavonoids (quercetin and myricetin), anthocyanidins (cyanidin and delphinidin) and flavan-3-ols (catechin) [1].

The phenolic compounds and flavonoids are widely distributed in food and are important constituents of the human diet [2]. This class of compounds is present in fruits, vegetables, cereals and olive oil.

Caffeic acid is found in all plants because it is an intermediate in the biosynthesis of lignin, one of the principal sources of biomass. The concentration of caffeic acid in raspberries, black currant, strawberries and berries is generally high. The caffeic acid is an antioxidant in vitro and also in vivo [3-5].

Many flavonoids are easily recognised as flower pigments in most angiosperm families [6-8]. From chemically point of view, flavonoids are polyphenolic compounds possessing 15 carbon atoms and two benzene rings joined by a linear three carbon chain [9].

Quercetin is a flavonol, a plant-derivated flavonoid found in the bark and rinds of many plants and fruits (peel apple, berries).

Quercetin has antihistaminic and anti-inflammatory properties and may act in the sense of protecting the body against cancer and heart diseases [10]. The chemical structures of quercetin and caffeic acid are shown in (Figure1) and (Figure2).

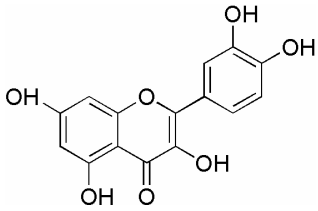

Figure 1. The chemical structure of quercetin.

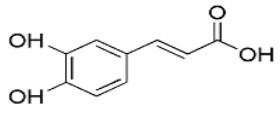

Figure 2. The chemical structure of caffeic acid.

Cranberry fruits (*Vaccinium macrocarpon)* are native from North America and are very close to bilberry and blueberry. These fruits represent an essential source of anthocyanins and polyphenols and they are under research for many benefits in the immune and cardiovascular system being used like anti–cancer agent in isolating prostate cancer cells. Cranberries are fruits with good antioxidant properties.

During the last decade, TLC and HPTLC have become important analytical techniques.

The advantages of using these methods are: simplicity, versatility, high velocity, specific sensitivity, simple sample preparation, short analysis time, low operating cost and possibility to simultaneously analyze the samples [11-14].

There are few articles in the literature regarding the usage of these techniques for identification and quantification of phenolic compounds [15-20].

In this work we described a method based on high performance thin layer chromatography for caffeic acid and quercetin determination from cranberry extract.

The experimental results allowed us to establish that the proposed method is rapid, simple and precise, as well sensitive and reproducible.

Materials and Methods

Reagents

The caffeic acid was purchased from Sigma-Aldrich (Schnelldorf, Germany), while the quercetin was purchased from Acros Organics **(**Nidderau**,** Germany). The HPTLC 60 F_{254} silica gel plates (20×10 cm, art.no.105642.0001) were supplied from Merck (Darmstadt, Germany).

For mobile phase preparation *n*-hexane (Merck), ethyl acetate (Merck), acetic acid (Merck) and hydrochloric acid 37% (Merck), were used.

Methanol, diphenylborinic acid aminoethylester and macrogol 400 were purchased from Sigma Aldrich (Schnelldorf, Germany). Ultra-pure water was produced by Synergy System (Millipore, Schwalbach, Germany).

Plate pre-treatment

Plate pre-treatment can be performed for a whole plate package in advance. The HPTLC silica gel plates were washed for pre-development with methanol.

The cleaned plates were dried and activated on the TLC Plate Heater III (CAMAG, Muttenz, Switzerland) at 120°C for 20 minutes.

The last step is necessary to completely remove all traces of the washing solvent.

In a desiccator, the active plate were cooled to room temperature and balanced with the relative humidity from the laboratory atmosphere. For temporary storage, the pre-washed plates were wrapped in aluminium foil.

Standard solution preparation

For stock solutions (1 mg/10 mL) preparation, caffeic acid and quercetin were dissolved individually in 10 ml of methanol.

For applications, we prepared a mixture of these stock solutions (600 µL): 300 µL of caffeic acid and 300 µL of quercetin.

Origin of samples

Cranberry (*Vaccinium macrocarpon*) extract was originated from USA and was obtained from Naturex (Köln, Germany).

Extraction of samples

The powdered samples were extracted from frozen berries using ethanol at 40-60 ºC without acid, during 12 hours.

Application

The solutions were sprayed as band (8 mm) with the Automatic TLC Sampler 4 (ATS4, CAMAG) using a 25 µL syringe allowing 7 tracks to be applied on one HPTLC plate of 10×10 cm (distance between bands 11.6 mm, distance from lower edge 8 mm and distance from the left side 15 mm).

For calibration of caffeic acid and quercetin on the plate (20 – 300 ng/band for standard-mix) were applied four different volumes (1, 5, 10, and 15 µL) from standard solution over a calibration range of 1:15.

From sample solutions were applied 2.5 µL. A constant application rate of 150nL/s was used. For routine analysis, each sample was applied three times on the plate.

Chromatography

Chromatography was performed on a 10×10 cm 60 F_{254} silica gel plate (Merck) and the development was performed in the Automated Developing Chamber 2 (CAMAG) at a relative air humidity of 25 ± 2 % with a mixture of n-hexane, ethyl acetate, acetic acid (5:3:1 *v/v/v/v*).

For a standardized separation, the plate activity was controlled for 4 minutes using a saturated potassium acetate solution (257.6g/100g H_2O) filled in the humidity control unit.

The migration distance was 45 mm from the lower plate edge and the migration time was 15 minutes. After the development, the plate was dried in a stream of cold air for 3 minutes.

Post-chromatographic derivatization

After chromatography, the plate was heated on the TLC Plate Heater III at 120 °C, during 5 minutes. The derivatization was performed with natural products – polyethylene glycol reagent (NP/PEG) in TLC Immersion Device III (CAMAG) using a vertical speed of 3 cm/s. The immersion time was 3 seconds.

After the derivatization, the plate was dried in cold air steam for 3 minutes. For natural products-polyethylene glycol reagent (NP/PEG) preparation, 2 g of diphenylborinic acid aminoethylester and 10 g of polyethylene glycol 400 were dissolved separately in 200 mL of ethyl acetate. Both solutions were stable for at least three months.

Documentation

Plate images were documented by TLC Visualizer Documentation System (CAMAG) consisting in a powerful high-resolution 12 bit CCD digital camera with outstanding linearity.

All images were captured with an exposure time of 30 ms under UV light at $\lambda = 254$ and $\lambda = 366$ nm, reflectance mode, respectively. The obtained data were processed with win CATS software, 1.4.7.2018 (CAMAG) version.

Quantification by densitometry

The densitometric evaluation was performed with a TLC Scanner4 (CAMAG) after peak height.

Fluorescence measurement of the derivatized plate with the Neu's reagent was recorded under UV light at λ = 366 nm.

Results and Discussions

The preliminary results of this study confirm the presence of caffeic acid and quercetin in *Vaccinium macrocarpon* extract.

Chromatographic method

Caffeic acid and quercetin were separated on the HPTLC 60 F_{254} silica gel plate (fluorescence indicator) prewashed with methanol and heated at 120 ºC for 20 minutes in order to remove all traces of the washing solvent.

For optimization, various combinations of mobile phases were used in order to obtain the best resolution. Caffeic acid and quercetin were best separated at a 5:3:1 (*v/v/v*) ratio and a migration distance up to 45 mm. In the second branch of the developing chamber were introduced 2.5 mL of hydrochloric acid 37 %.

Caffeic acid was identified as the fluorescent green spot and quercetin as the orange spot.

The retention factor (R_f) values of this two compounds were 0.33 and 0.44 respectively. The results obtained for separation and evaluation after derivatization are presented in (Figure 3) and (Figure 4). On one plate, the same sample was applied three times.

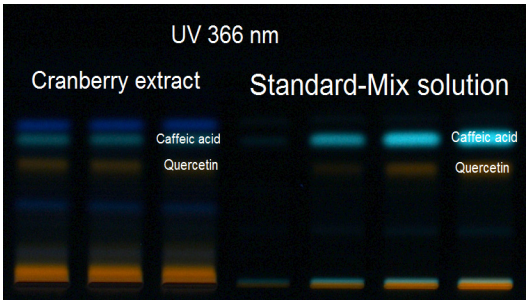

Figure 3. Separation of caffeic acid and quercetin from cranberry extract – documentation after derivatization in UV light at λ = 366 nm.

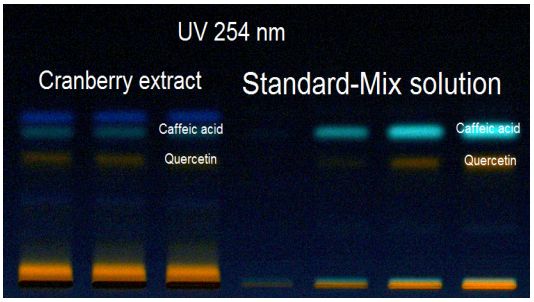

Figure 4. Separation of caffeic acid and quercetin from cranberry extract – documentation after derivatization in UV light at λ = 254 nm.

Sample preparation

One gram (1 g) of extract was weighed in a 10 ml dark volumetric flask fitted with a ground-glass stopper and sonicated on water bath with 10 ml of mixture methanol/water (4:1) for 30 minutes at room temperature.

The resulting solution was filtered through 0.45 µm pore-size cellulose filters and stored at -20 ºC.

Quantification

After development with *n*-hexane, ethyl acetate and acetic acid and fluorescence measurement at λ = 366 nm, the quantification was performed by means of peak height evaluation.

The 3D display of HPTLC chromatogram for cranberry extract is presented in (Fig. 5).

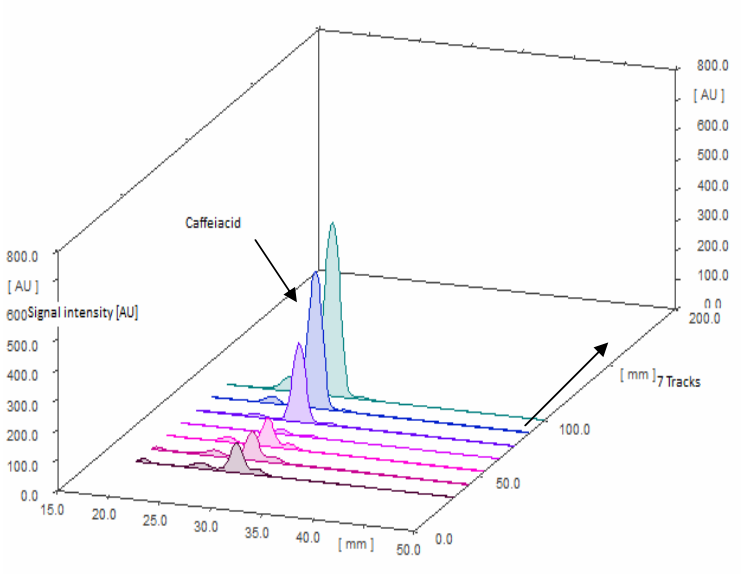

Figure 5. The 3D display of HPLTC chromatogram of caffeic acid from cranberry extract samples – fluorescence scan at λ = 366 nm

For example, a 4-point calibration in the 20-300 ng/band range standard mixture was suited to analyse a wide range of caffeic acid and quercetin finding in cranberry samples.

Starting with the quantification limit, the calibration curves of caffeic acid and quercetin are showed in (Figure 6) and (Figure 7).

The calibrations presented good performance characteristics, with correlation coefficient values between R = 0.99984 and R = 0.99972 and relative standard deviation *%RSD* = 1.6 % and 1.95 % measured by peak height, in polynomial regression ($Y = -0.004 \cdot X^2 + 3.369 \cdot X - 47.694$) for caffeic acid and linear regression ($Y = 0.121 \cdot X - 3.271$) for quercetin.

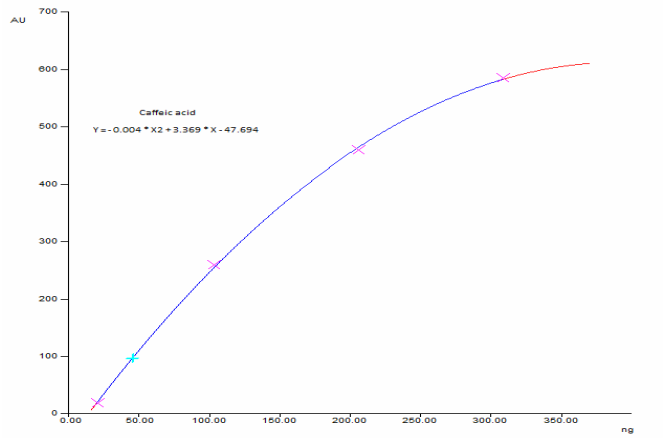

Figure 6. Caffeic acid polynomial calibration after via peak height by fluorescence at λ = 366 nm with a 0.99984 value for the correlation coefficient.

The performance data of the standardized HPTLC method for caffeic acid and quercetin analysis in cranberry extracts are shown in (Table 1).

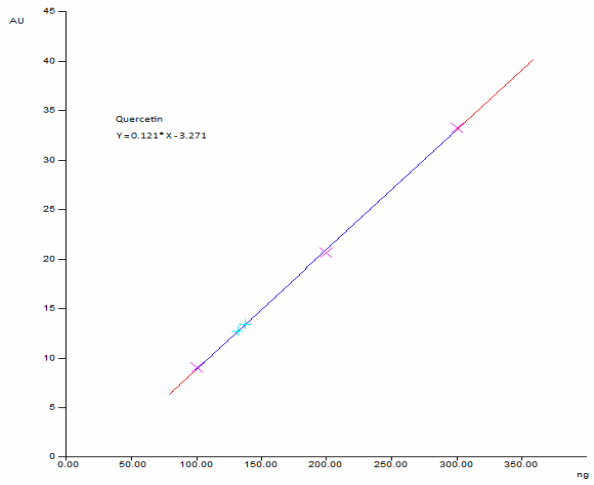

Figure 7. Quercetin linear calibration after peak height by fluorescence at λ = 366 nm with a 0.99972 correlation factor.

Table 1. Performance data of HPTLC method for caffeic acid and quercetin determination in cranberry extract

Standard	**R_f**	**Regression mode**	**Conc (ng/band)**	**Conc (%)**
Caffeic acid	0.44	polynomial	31.17	0.012
Quercetin	0.33	linear	134.82	0.054

Conclusion

This work presents a convenient HPTLC method for quercetin and caffeic acid determination from cranberry fruits.

Using *n*-hexane, ethyl acetate and acetic acid (5:3:1, *v/v/v*), as mobile phase, good separations were rapidly obtained.

The correlation coefficient was ≥ 0.9997. Statistical analysis showed good repeatability and selectivity for the analysis.

The results provided by our study showed that cranberry is an essential source of antioxidant compounds and could play an important role in improving antioxidant intake in the human diet.

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