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SHORT COMMUNICATION

A high-performance thin-layer chromatographic method for chlorogenic acid and hyperoside determination from berry extracts

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Abstract

The present study investigates a quantitative high performance thin layer chromatographic (HPTLC) method for chlorogenic acid and hyperoside determination from berry extracts. Chlorogenic acid and hyperoside were extracted using a methanol and water mixture and separated on a HPTLC 60F_{254} silica gel plates with ethyl acetate, acetic acid, formic acid and water 10:1.1:1.1:2.3 (v/v/v/v) as mobile phase within 35 minutes. After chromatography, the plates were sprayed with natural products (polyethylene glycol reagent (NP/PEG)). Densitometric analysis of these compounds was performed via fluorescence, under UV light at λ = 366 nm. The R_f of chlorogenic acid and hyperoside was 0.74 and respectively 0.85. Calibration data revealed good linear and polynomial relationships (r = 0.99996 and 0.99999) between peak height area and chlorogenic acid, respectively hyperoside concentration. Based on the results obtained in this study, the investigated method, can be used for rapid routine analysis of chlorogenic acid and hyperoside.

Keywords: berry extract, chlorogenic acid, hyperoside, HPTLC

Introduction

Medicinal plants have played an essential role in the development of human culture. These plants are used in human or veterinary practice for therapeutic or prophylactic purposes due to their qualities. Herbs are available in many forms including fresh, dried, capsules, tablets or bottled in liquid form.

Berries represent a particularly rich source of anthocyanins, flavonoids and polyphenols. They also contain other bioactive substances like C and E vitamin. Anthocyanins have benefits in heart health, brain health and could play an important role in cancer risk reduction. Phenolic compounds like caffeic, chlorogenic, ferulic, sinaptic acid also have good antioxidant properties and are considered an important part of the defense mechanism [1].

Together with caffeic, p-cumaric and ferulic acid, chlorogenic acid is an ester of hydroxycinnamic acid with quininic acid [2,3]. This acid is an important biosynthetic intermediate and has good antioxidant and inhibitory properties.

Chlorogenic and caffeic acids are in vitro antioxidants and fight against diabetes and cardiovascular disease [4,5].

Hyperoside is a flavone found in certain herbs with strong antioxidant activity. This compound has a variety of pharmacological effects, including anti-inflammatory, anti-viral,

anti-oxidative [6,7]. The chemical structures of hyperoside and chlorogenic acid are shown in (Figure 1) and (Figure 2).

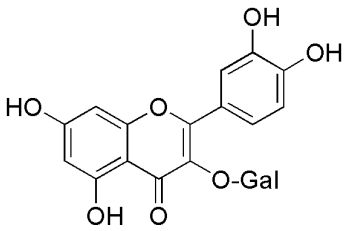

Figure 1. The chemical structure of hyperoside.

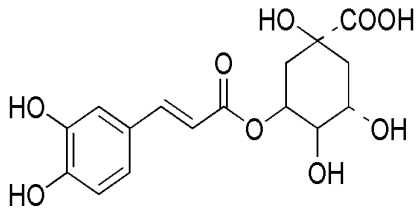

Figure 2. The chemical structure of chlorogenic acid.

Bilberries fruits (*Vaccinium myrtillus)* are native from Europe and have similar health benefits to blueberries. Bilbberries are a good source of vitamins, minerals, fibers and pigments based antioxidants. These fruits contain antioxidants (anthocyanins, phenolic acids, flavonoids, tannins, C vitamin), which help to neutralize free radicals that destroy collagen in cells and tissues [8].

The C vitamin repairs blood vessels and cells and stimulates the immune system activity. Bilberry fruits are useful in the prevention and healing of varicose veins and urinary tract infections. When are used as tea or juice, bilberries can fight against digestive problems such as nausea.

Cranberry fruits (*Vaccinium macrocarpon)* are native from North America. These fruits are used in food industry for juice preparation and small quantities are sold fresh to the consumers. Cranberry fruits contain significant amounts of flavonoids, polyphenolic compounds, C vitamin, manganese and potassium [9].

In smaller amounts we can find B6 vitamin, pantothenic acid, A vitamin, thiamine, riboflavin and niacin. This fruits are rich in antioxidants, due to their content of proanthocyanidins.

Antioxidants can neutralize free radicals and reduce some of the damages that they are causing [10]. Cranberries are used to prevent and treat urinary tract infections, diabetes and cancer. [11-13].

Blueberry fruits *(Vaccinium cyanococcus)* are beloved for their sweet taste, their spicy flavor and their indigo blue color.

The main benefit of these fruits is related to the high level of anthocyanins (delphinidin, cyanidin, malvidin and peonidin), hydroxycinnamic acids (caffeic, ferulic and cumaric), flavonoids (kaempferol, quercetin and myricetin), tannins and resveratrol, which play an important role in reducing the risk of cancer, inflammation and diseases appearance [14-18].

The manganese and C vitamin are the main nutrients found in blueberries. These fruits are a good source of E, K vitamin and dietary fibers.

HPTLC methods present many advantages including: simple sample preparation, low operating cost, short analysis time and simultaneous analysis of several samples. Several articles regarding the identification and quantification of phenolic compounds using this technique are available in the literature [19-25].

The aim of this study was to identify and determine the chlorogenic acid and hyperoside from cranberry, bilberry and blueberry samples, using a high performance thin layer chromatography method, which proved to be fast and accurate.

Materials and Methods

Reagents

Chlorogenic acid and hyperoside were purchased from Carl Roth (Karlsruhe, Germany), while rutin 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 we used ethyl acetate (Merck), acetic acid (Merck) and formic acid (Merck). 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 full plate package in advance. The HPTLC silica gel plates were washed by pre-development with methanol.

The cleaned plate 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

For stock solutions preparation (1 mg/10 mL), chlorogenic acid, hyperoside and rutin were individually dissolved in 10 ml methanol.

For application was prepared a mixture (900 µL) from these stock solutions as follows: 300 µL of chlorogenic acid, 300 µL of hyperoside and 300 µL of rutin.

Origin of samples

Bilberry (*Vaccinium myrtillus*) and blueberry (*Vaccinium cyanococcus*) extracts were originated from Europe and purchased from BerryPharma (Leichligen, Germany) and respectively from Huisong Pharmaceuticals (Zhejiang, China). Cranberry (*Vaccinium macrocarpon*) extract was originated from USA and purchased 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 in the form of 8 mm band using an Automatic TLC Sampler 4 (ATS4, CAMAG) and a 25 µL syringe. On one HPTLC plate with the following characteristics: 20×10 cm, distance between bands 11.6 mm, distance from lower edge 8 mm, distance from the left side 30 mm, were applied 13 tracks.

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

For hyperoside determination on the plate was applied 1 µL of cranberry solution, 15 µL of blueberry solution and 12 µL of bilberry solution.

For chlorogenic acid determination on the plate was applied 1.5 µL of cranberry solution, 10 µL of blueberry solution and 8 µL of bilberry solution.

For routine analysis each sample was applied three times on the plate. A constant application rate of 150 nL/s was used.

Chromatography

The chromatography was performed on a 20×10 cm 60 F_{254} silica gel and the development was performed in the Automated Developing Chamber 2 (CAMAG) at a relative air humidity of 25 ± 2 % with a mixture of ethyl acetate, acetic acid, formic acid and water (10:1.1:1.1, 2.3 *v/v/v/v*).

For separation standardization, it was used a humidity controller that was filled with saturated potassium acetate solution (257.6g/100g H_2O) in the humidity control unit.

The migration distance was 70 mm and the migration time was 35 minutes.

After the development, the plate was dried in a cold air stream for 3 minutes.

Post-chromatographic derivatization

After chromatography, the plate was heated on the TLC Plate Heater III at 120 °C, for 5 minutes.

The derivatization was performed with polyethylene glycol reagent (NP/PEG), a natural product, in TLC Immersion Device III (CAMAG) at a vertical speed of 3 cm/s. The immersion time was 3 seconds. After the derivatization, the plate was dried in cold air steam during 3 minutes.

For natural product preparation, namely polyethylene glycol reagent (NP/PEG), 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.

Evaluation

Plate images were evaluated with a TLC Visualizer Documentation System (CAMAG) using a high-performance 12 bit CCD digital camera with outstanding linearity.

All images were captured with an exposure time of 30 ms under UV light at λ = 254 and λ = 366 nm, in reflected way. The obtained data were processed using a winCATS software, 1.4.7.2018 (CAMAG) version.

Densitometry quantification

Densitometric evaluation was performed by a TLC Scanner 4 (CAMAG) after peak height. Fluorescence measurement of derivatized plate with polyethylene glycol reagent (NP/PEG) – natural product was recorded under UV light at λ = 366 nm.

Results and Discussions

The preliminary results of this study confirmed the presence of chlorogenic acid and hyperoside in methanol-water extracts of cranberry, bilberry and blueberry.

Chromatographic method

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

For optimization, various combinations of mobile phases were used in order to obtain best resolution and reproductibility of the peaks.

The chlorogenic acid and hyperoside were best separated at a 10:1.1:1.1:2.3 (*v/v/v/v*) ratio for a 70 mm migration distance.

The fluorescent blue spot corresponds to chlorogenic acid and the orange spot to hyperoside (Figure 3).

The retention factor, R_f, values for this two compounds are 0.74 and 0.85, respectively.

The separation and evaluation after derivatization in UV light at λ = 366 nm are presented in (Figure 3). On one plate, the same sample was applied three times.

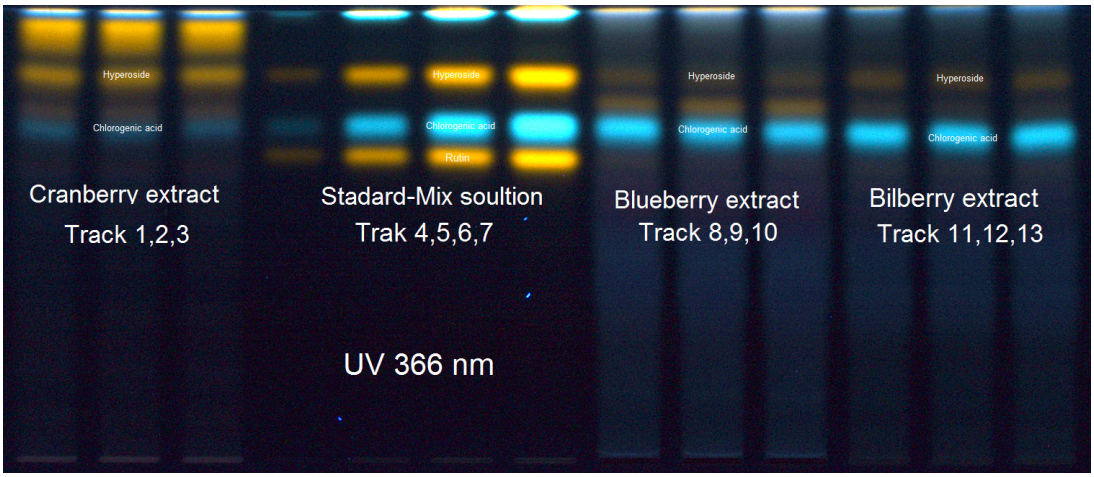

Figure 3. Separation of chlorogenic acid and hyperoside from berry extracts – examination after derivatization in UV light at λ = 366 nm.

Sample preparation

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

The resulting solution was filtered through cellulose filters with a 0.45 µm pore size. For blueberry and bilberry solution were obtained 0.005 g/mL and respectively, 0.0004 g/mL concentrations. The solutions were stored at -20 ºC.

Quantification

After derivatization with polyethylene glycol reagent (NP/PEG) - natural product and fluorescence measurement at λ = 366 nm, quantification was performed by peak height evaluation. The 3D display of HPTLC chromatogram for berry extracts is shown in (Figure 4).

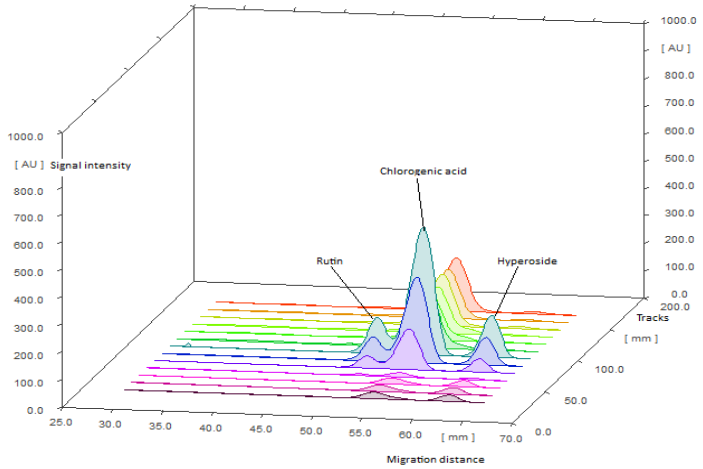

Figure 4. The 3D dispay of HPTLC chromatogram of chlorogenic acid and hyperoside from berry samples – fluorescence scan at λ = 366 nm.

The method was validated for instrumental precision, repeatability, specificity and linearity.

The quantification data for chlorogenic acid and hyperoside from cranberry, blueberry and bilberry extracts are shown in (Table 1).

Table 1. Quantification data for chlorogenic acid and hyperoside from cranberry, blueberry and bilberry extract.

Sample	Compound	Applied volume (µL)	Solution concentration (g/mL)	Sample concentration (ng/band)	Evaluated by
Cranberry extract	Hyperoside	1	0.1	53.54	height
	Chlorogenic acid	1.5		40.82	height
Blueberry extract	Hyperoside	15	0.005	14.56	height
	Chlorogenic acid	10		87.94	height
Bilberry extract	Hyperoside	12	0.0004	21.28	height
	Chlorogenic acid	8		90.79	height

For example, a 4-point calibration in the 20 – 300 ng/band range of standard mixture was suited to analyze a wide range of chlorogenic acid and hyperoside findings in berry samples.

Starting with the quantification limit, the calibration curves of chlorogenic acid and hyperoside are showed in (Figure 5 and Figure 6).

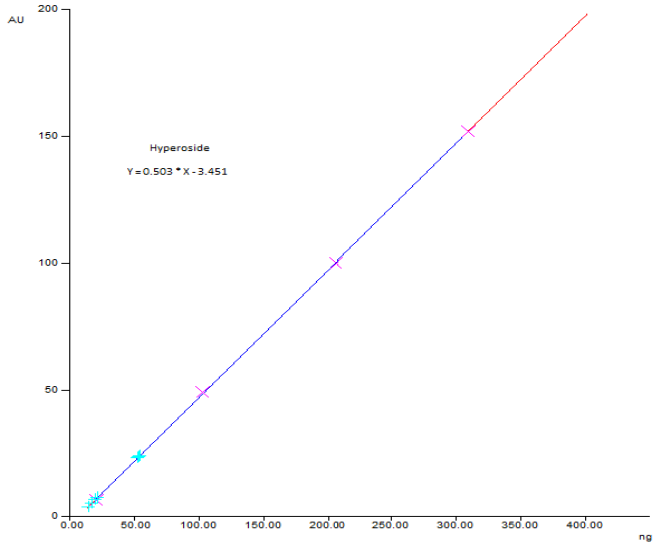

Figure 5. Hyperoside linear calibration performed after peak height in UV light at λ = 366 nm with a 0.99996 correlation coefficient.

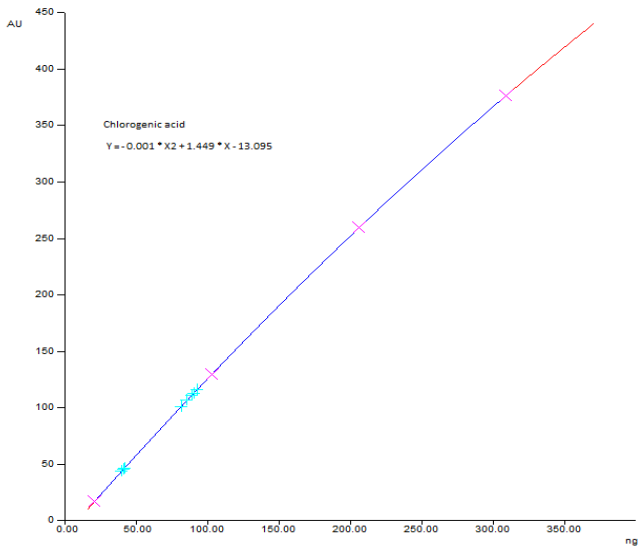

Figure 6. Chlorogenic acid polynomial calibration performed after peak height in UV light at λ = 366 nm with a 0.99999 correlation coefficient.

The calibrations presented good performance characteristics, with correlation coefficient values, R, between 0.99999 and 0.99996 and relative standard deviation *% sdv* = 0.03 % and 0.85 % measured by peak height, in polynomial regression for chlorogenic acid and in linear regression for hyperoside.

The performance data of HPTLC method for the determination of chlorogenic acid and hyperoside in cranberry, blueberry and bilberry extract are shown in (Table 2).

Table 2. Performance data of HPTLC method for chlorogenic acid and hyperoside determination in cranberry, blueberry and bilberry extract.

Compound	**R_f**	**Equation**	**Regression mode**	**Correlation coefficient (r)**	**Relative standard deviation (sdv)**
Chlorogenic acid	0.74	$Y= - 0.01X^2 + 1.449X – 13.095$	polynomial	0.99999	0.03
Hyperoside	0.85	$Y = 0.503X – 3.451$	linear	0.99996	0.85

Sample analysis

The amounts of chlorogenic acid and hyperoside varied from species to species and are shown in (Table 3).

The chlorogenic acid concentration was maximum in Vaccinium myrtillus (2.84%) and minimum in Vaccinium macrocarpon (0.053%).

The hyperoside concentration was maximum also in Vaccinium myrtillus (0.44%) and minimum in Vaccinium Cyanococcus (0.019%). Rutin was not found in samples.

Table 3. Amounts (%) of chlorogenic acid and hyperoside in cranberry, blueberry and bilberry extracts.

Species	Chlorogenic acid	Hyperoside
Vaccinium macrocarpon (cranberry extract)	0.027	0.053
Vaccinium Cyanococcus (blueberry extract)	0.175	0.019
Vaccinium myrtillus (bilberry extract)	2.836	0.443

Conclusions

This paper presents a convenient HPTLC method for hyperoside and chlorogenic acid determination from bilberry, blueberry and cranberry extracts.

Using ethyl acetate, formic acid, acetic acid and water (10:1.1:1.1:2.3, *v/v/v*), as mobile phase, rapidly good separations were obtained.

The correlation coefficients, R, were ≥ 0.99996. Statistical analysis showed good reproductibility and selectivity for the determinations.

The results provided by our study show that berries are an essential source of antioxidant compounds which play an important role in stopping or preventing damage to the body cells.

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