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Application of micellar electrokinetic capillary chromatography for quantitative analysis of quercetin in plant materials

A simple method for the routine determination of quercetin in biological samples was developed. The method consists of hydrolysis of bonded quercetin, its isolation and preconcentration on solid-phase extraction (SPE) column and a final analytical step using micellar electrokinetic capillary chromatography. The working range, linear range, the limit of quantification, and the measurement uncertainty were tested in validation. The method is suitable for quercetin determination in fruit and vegetable samples.

Keywords: Flavonoids / Quercetin / Micellar electrokinetic capillary chromatography / Plant material
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1 Introduction

Flavonoids represent a large group of plant phenols. Currently more than 4000 flavonoid compounds are known and new ones are being found. Flavonoids are derived from heterocyclic 2-phenylbenzopyrone. Commonly all three cycles are substituted by hydroxyl groups or methoxy groups and discrete derivatives differ in the stage of substitution and oxidation. Natural flavonoids occur usually in the *O*-glycosidic form. Free aglycones are seldom found. Hydrolysis of glycosides resulting in the increase of aglycone's concentration can occur especially during the technological treatment of foods, at higher temperatures and under acidic conditions [1].

Flavonols, the subgroup of flavonoids represented mainly by quercetin (Fig. 1) and kaempferol, are abundant in fruits and vegetables [2–4]. Flavonoids act as an important component of antioxidative systems preventing from the formation of lipid peroxiradicals, eliminating free oxygen radicals, binding and inactivating some metal cations (iron, copper) [5, 6]. The antioxidative activity of flavonoids depends on the number and the position of hydroxyl groups as well as their glycosylation in the molecule.

Optimum radical-scavenging activities have been found for an *o*-dihydroxy structure in the B-ring, 2,3 double bond, 4-oxo function in the C-ring, and 3- and 5-OH groups in the A- and C-rings [7]. Flavonols combine these features. Showing these properties, flavonoids can prevent diseases originating in oxidative damage of biological structures (cardiovascular diseases, atherosclerosis) [7]. Appropriate structure of alimentation with elevated contents of flavonoids could help in the prevention and in combating these diseases [5]. For the flavonoids analysis, methods enabling separation of complex mixtures of similar compounds should be used. Usually HPLC is being applied [8]. The use of capillary zone electrophoresis (CZE) is frequent [9]. Prior to HPLC or CZE analysis flavonoids are either extracted from samples using an organic solvent [10] or hydrolysed [4, 11]. Acid hydrolysis enables the liberation of all aglycones from respective flavonoid glycosides.

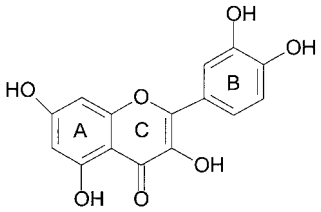

Figure 1. Structure of quercetin

Capillary electrophoresis and especially micellar electrokinetic capillary chromatography (MEKC) are methods suitable for analysis of complex mixtures of natural organic substances having a similar chemical structure. As flavonoids and their aglycones are weakly polar compounds, MEKC seems to be a more convenient method for the determination. This method enables optimum determination of flavonoid compounds in food samples or drugs. Filtered liquid samples are sometimes applied directly to the analyser [12]. Vine samples [13] as well as solid samples [14] can be extracted by diethyl ether or methanol, respectively. Ferreres *et al.* [15] proposed an analysis of honey by preseparation of flavonoids on Amberlite. Solid-phase extraction (SPE) on C-18 columns

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Abbreviation: TBHC, *t*-butylhydroquinone

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followed by elution of preconcentrated flavonoids by methanol is probably the most acceptable way of preseparation [8].

Due to the fact that there exists a plethora of flavonoid compounds, it is very likely, that most of natural samples would contain a characteristic flavonoid pattern. The determination of each component of such mixture would be difficult, especially with respect to the standard compounds availability. As flavonoids contain only several kinds of aglycones, there might be a solution of this task by converting glycosides to the respective aglycones by acid hydrolysis followed by the determination of the aglycones by MEKC. The total amount of aglycones would correspond to the total amount of all kinds of flavonoids in given material. The aim of this work was to examine the possibilities of the determination of quercetin in natural samples. The method is based on the acid hydrolysis of sample followed by the MEKC determination.

2 Materials and methods

2.1 Chemicals

All chemicals were of analytical grade. Quercetin (Sigma-Aldrich, Deisenhofen, Germany); sodium tetraborate, *t*-butylhydroquinone (TBHCH) (Fluka, Buchs, Switzerland); acetylsalicylic acid, boric acid, phosphoric acid, benzoic acid, tungstophosphoric acid, methanol, sodium hydroxide (Lachema, Brno, Czech Republic); 1-naphthylacetic acid (Spolana, Neratovice, Czech Republic); salicylic acid (Merck, Darmstadt, Germany); sodium dodecyl sulfate (SDS) (Sigma Chemicals, St. Louis, MO, USA). Deionized water was prepared with Premier (Premier Systems, Phoenix, AZ, USA). For pH measurements a LogR-meter m.370 (Orion Res. Inc., Boston, MA, USA) was used. Glas filters Nr. S7 were by Filpap (Hostiné, Czech Republic). The SPE columns LiChrolut RP-18, RP-18 end-capped, NH_2, CN, Si (Merck), LC-Ph (Supelco, Bellefonte, PA, USA) were flushed with 3×2.5 mL methanol and 4×2.5 mL water prior to use.

2.2 Standard solutions

Solutions of quercetin standard (1 mg/mL) and of the internal standard 1-naphthylacetic acid (2 mg/mL) were prepared by dissolving the substance in 100% methanol. These stock solutions were stored in the refrigerator (4°C) and were used after appropriate dilution. The stability of standard solutions was tested by their spectra measurements (200–350 nm) every week. It was found, that under above-mentioned conditions the solutions are stable at least for 6 months.

2.3 Samples

Samples of fruits and vegetables were obtained from a private producer from central Bohemia. We used apples (variety "Prusvitne letni"), early white cabbage, and cauliflower. Natural products were not chemically treated, fertilized only with natural fertilizers, and harvested at maturity optimal for consumption. Material for analysis was washed and uneatable parts (stalks, cores *etc.*) were discarded. Samples were cut to 5 mm thick pieces and freeze-dried (24 h, –46°C, 0.25 mbar). The dried material was stored in the freezing box (–18°C) in closed containers.

2.4 Apparatus

Analyses were carried out on Spectraphoresis 2000, a fully automated system for CZE equipped with a multiwavelength UV-Vis scanning detector (Thermo Separation Products, Fremont, CA, USA). Separations were achieved in plain fused-silica capillary of 70 cm length (67 cm effective length to the detector) $\times$ 75 μm ID (CElect FS75 CE column; Supelco). Data processing was performed using Spectacle and PC 1000 CE software Version 3.0. Injection of the samples was achieved by a 2 s vacuum application. Approximative volume of the sample injected was 12 nL. The applied voltage (+20 kV) resulted in an electrophoretic current of 30 μA and the temperature around the capillary was maintained constant by the Peltier system at 25 $\pm$ 0.01°C. The wavelength of detection was set at 270 nm. The separation buffer consisted of 10 mM boric acid, 10 mM sodium tetraborate, 20 mM SDS, 15% methanol, pH 9.2. A new capillary was washed for 5 min with 1 M NaOH at 45°C, 5 min with 0.1 M NaOH at 45°C, and 20 min with water at 25°C. After each sample injection the capillary was postwashed for 2 min with 0.1 M H_3PO_4 at 45°C, 2 min with 0.1 M NaOH at 45°C, and 5 min with water at 25°C.

2.5 Sample preparation

The method of hydrolysis is based on literature [3]. For hydrolysis, 0.5 g of grinded freeze-dried sample in a flask is mixed with ascorbic acid solution (80 mg in 7.5 mL water). When the sample is imbibed with the liquid, 12.5 mL of methanol and 5 mL of 6 M HCl are added. Samples are boiled in a water bath under reflux cooler for 2 h, the temperature of the water bath is maintained on 90°C. After 10 min of cooling to the room temperature, the hydrolysed sample is neutralized by the addition of 2 g $NaHCO_3$ and transferred to a beaker by 7.5 mL of methanol and 100 mL of water. The volume in the beaker is made up to 200 mL by water and pH of the mixture is adjusted to pH 3.0 by the saturated $NaHCO_3$ solution. To

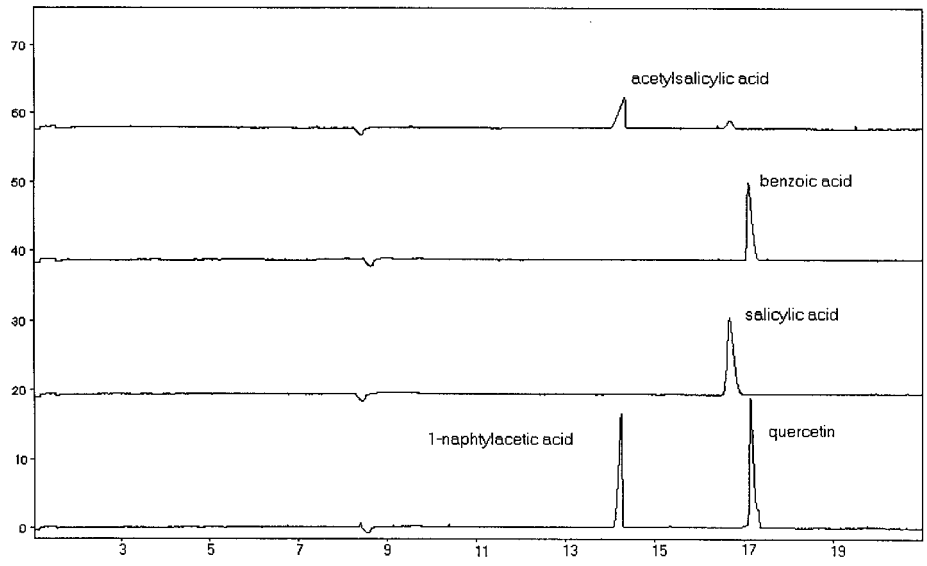

Figure 2. Electropherogram of four tested internal standards and quercetin. Separation conditions: buffer, 10 mM boric acid, 10 mM sodium tetraborate, 20 mM SDS, 15% v/v methanol; 25°C, voltage, +20 kV; wavelength, 270 nm; hydrodynamic injection, 2 s.

this sample 2 mL of 5% m/m tungstophosphoric acid solution are added. After 15 min the mixture is filtered through glass fibre filters. The cake is rinsed with 5 mL of methanol. The filtrate is made up to 500 mL by water. SPE columns (RP-18) were used for isolation of quercetin. Before use each column was washed with 4×2.5 mL of methanol and 4×2.5 mL of water. The flow rate of the sample through the column was set to 15 mL/min. Sample was applied onto the column, then the column was washed with 4×2.5 mL of water and dried for 15 min by air aspiration. The SPE column was eluted by 2×0.7 mL of methanol. The resulting solution on the vial is spiked with 0.1 mL of the internal standard solution (1-naphthylacetic acid, 2 mg/mL in methanol). This solution represents the sample for MEKC analysis.

3 Results and discussion

3.1 Optimization of the determination

In capillary electrophoresis, the hydrodynamic injection is probably the most frequently used way of sample introduction. The reproducibility of this kind of injection is sometimes not satisfactory. The use of internal standard helps to overcome the above-mentioned problems. Among compounds possessing similar chemical and analytical properties as quercetin in selected systems, the benzoic, salicylic and 1-naphthylacetic acids seemed to be the most promising. Further experiments showed that the 1-naphthylacetic acid has optimal properties (Fig. 2).

For the quercetin determination the borate buffer (pH 9.2) was chosen. When real samples were analyzed in this plain borate buffer, interference of the analyte and other constituents of the sample was observed. For improving the partition effect, the organic modifier is sometimes beneficial [15, 16]. Four different concentrations (0, 5, 10 and 15%) of methanol were tested. The addition of methanol (15% v/v) to the borate buffer improved the resolution. The total analysis time does not exceed 20 min. The hydrolysis of the total flavonoid glycosides results in the liberation of quercetin which is further isolated and preconcentrated on SPE columns. The sample is then ready for MEKC determination.

3.2 Optimization of the sample preparation

The sample subdued to hydrolysis should be spiked with an antioxidant to prevent the analyte from the oxidative degradation. We tested the antioxidative properties of TBHCH [3] and ascorbic acid [17]. TBHCH was excluded due to its interference with the analyte in MEKC. The ascorbic acid did not show any negative properties in the MEKC system. The hydrolysed sample contains an abundance of colloid particles. These substances are not removed by filtration and in further step they clog SPE columns. Colloid particles must be precipitated by the proper agent and together with unsoluble fraction removed by filtration. For this purpose, we examined the effect of the Carres reagent and tungstophosphoric acid [18] followed by vacuum filtration through paper filter or glass fibre filters. The Carres reagent was suitable for filtration through paper filters, but the recovery of quercetin was poor, probably due to quercetin adsorption on paper. Samples treated with the Carres reagent clogged the glass fibre filters. The best results were obtained with tungstophosphoric acid and filtration through glass fibre filters. Six types of SPE columns were proved (LiChrolut

RP-18, RP-18 end-capped, NH_2, CN, Si (Merck), LC-Ph (Supelco)). The best recovery of quercetin was achieved on RP-18 columns. Table 1 describes the recovery of quercetin on several types of SPE column.

Table 1. Recovery of quercetin on six SPE columns

SPE column	RP-18	RP-18 E	NH_2	CN	SI	Ph
Recovery (%)	79	33	0	3	0	62

Columns: RP-18, LiChrolut RP-18 Merck; RP-18 E, LiChrolut RP-18 end-capped Merck; NH_2, LiChrolut RP-18 Merck; CN, LiChrolut RP-18 Merck; Si, LiChrolut RP-18 Merck; Ph LC-Ph Supelco

3.3 Method validation

3.3.1 Working range

One of the important objectives was to confirm the suitability of the method for the intended purposes. In this part of our work, the following parameters were taken into consideration: working range, linear range, limit of quantification, and measurement uncertainty. The working range was proposed for the concentration range of 3–100 μg/mL according to the expected quercetin contents in samples [4, 11]. The ratio of quercetin peak area and the peak area of the internal standard was considered as analytical response. Conformably to ČSN ISO 8466-1, the values of residuals of the standard deviation for linear (s_{y1} = 0.0159) and nonlinear (s_{y2} = 0.0167) calibration functions do not significantly differ. Moreover, the non linear function does not represent an important improvement in data fitness; the use of the linear function is acceptable.

3.3.2 Limit of detection

The limit of detection was determined by triplication of the average standard deviation of ten independent determinations of samples with quercetin content approaching the expected detection limit. For the assessment of the quantification limit the average standard deviation was decupled. The assessment of the detection limit and the quantification limit are 0.5 μg/mL and 1.5 μg/mL, espectively. For practical use, the quantification limit is considered to be 2 μg/mL.

3.3.3 Measurement uncertainty

The measurement uncertainty was calculated as duplicate of the relative standard deviation of ten independent determinations of samples with quercetin contents approaching the lower (3 μg/mL) and the upper (100 g/mL) limit of the working range. The measurement uncertainty was 5.3 and 4.5% for the lower and the upper limit of working range, respectively. From these values the measurement uncertainty was totalized and considered to be 10% [19].

3.3.4 Recovery

Due to the number of steps preceding analysis it is very likely, that some losses of analyte during the sample preparation may occur. For the recovery calculation the spiked matrix samples were used. According to literature data [11] confirmed by our observations, homogenized cauliflower samples contained the quercetin content below the detection limit. Samples of 0,5 g of freeze-dried cauliflower were spiked with quercetin standard in concentrations covering the whole working range of the method. Spiked samples were processed by the same way as other natural samples. The average value of the 40 samples recovery was 75% with the an RSD value of 4.4%. We did not observe any relation between the amount of the added quercetin and the recovery. When searching the reasons for the losses of quercetin in the sample preparation steps, we found that approximately 8% of quercetin were not eluted from the SPE column by methanol. The next 17% is not probably sorbed on the SPE column. The sorption on the walls was not observed. Due to the fact, that the recovery is constant over the whole calibration range, the constant recovery of 75% is taken in to consideration in all calculations.

3.3.5 Robustness

The method is robust enough if small differences in the standard method do not significantly affect the results. Based on the practical experience with the method development, the following parameters were assessed as critical: the time of column drying, sample pH before precipitation and filtration, the volume of solution used for precipitation, and the shelf life of the extract prior analysis.

(i) Residual moisture: Model samples were analyzed by standard way. The water content was determined by gravimetric analysis after 5, 10, 15 and 20 min of drying. After 10 min of drying, the column contains about 40 mg of water. It was proved that this amount had no significant influence on the quercetin recovery. The 15 min drying time in the standard method is sufficiently long.

(ii) Shelf life of the extract: Extracted quercetin was stable in the refrigerator (4°C). No decrease of analyte was observed in four weeks.

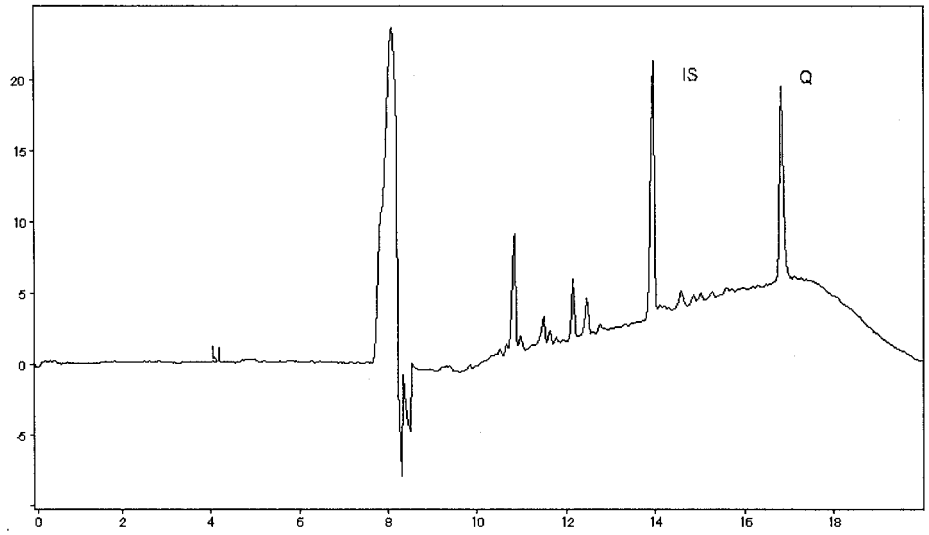

Figure 3. Electropherogram of fruit sample (apple "Prusvitné letní"). Separation conditions as described in Section 2.4.

(iii) The sample pH value: Before precipitation, the pH value is set to 3.0. The experiments revealed that fluctuations of pH within 2.8–3.2 do not exercise apparent influence over the quercetin recovery. The precision of pH meters enables easily to maintain pH within that safe range.

(iv) The coagulant volume: The coagulant volume might affect the analysis results. The $\pm$ 0.1 mL volume variations of this additive did not affect the quercetin recovery. As the pipette error is less than $\pm$ 0.02 mL, we suppose this method to be robust enough to the fluctuation of the coagulant solution.

3.4 Determination of quercetin in real samples

Biological material (fruits, vegetables) processed by the described method gave extracts, which were analyzed by the MEKC method. The quercetin content was calculated from the analytical response and the equation of the calibration function. The calibration curve is periodically updated for each series of samples. Data for calibration were acquired from analyses of methanolic solutions containing quercetin in concentrations of 0, 3, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 μg/mL. Each solution contained 200 μg/mL of internal standard. The ratio of quercetin peak area and the peak area of the internal standard was the relative analytical response. For the measured data the least squares line ($y = a + bx$) was calculated (y, analytical response; x, concentration of quercetin in the extract).

The concentration of quercetin in the extract was calculated as follows:

$$x = (P_Q/P_{IS} - a)/b \quad (1)$$

where a, b are the coefficients of the calibration line, P_Q is the peak area of quercetin, P_{IS} is the peak area of internal standard, and x is the concentration of quercetin in the extract (μg/mL). This concentration was recalculated with respect to the dry matter content in the sample:

$$c = x \cdot V/m \cdot 100/z \quad (2)$$

where c is the quercetin content in the sample (mg/kg of dry matter), x is the concentration of quercetin in the extract calculated from the calibration curve (μg/mL), V is the volume of the extract (mL), m is the mass of the freeze-dried sample (g), and z is the recovery (75% considered) (%). The results are easily convertible to the fresh weight values. Two examples of electropherograms of biological samples are shown in Figs. 3 and 4.

The method described in this paper was used for samples of apples, cabbage, and cauliflower. The cauliflower samples did not contain detectable amounts of quercetin, which is consistent with literature data [11]. This was the well-founded reason for using cauliflower as matrix sample. The quercetin content in apples was found to be 33 $\pm$ 3 mgkg^{-1} of fresh weight, which is in accordance with literature data (36 $\pm$ 19 mgkg^{-1} [11]). The quercetin content in cabbage was 7 $\pm$ 1 mgkg^{-1}. The higher value of 110 mgkg^{-1} in literature [11] can be explained by the difference between the growing season of cabbage samples. Contrary to Hertog *et al.* [11], our samples of cabbage were cultivated in early spring in greenhouse. The method will further be used for a large-scale study of quercetin content in fruits and vegetables in the human diet with respect to the importance of flavonoids for human nutrition.

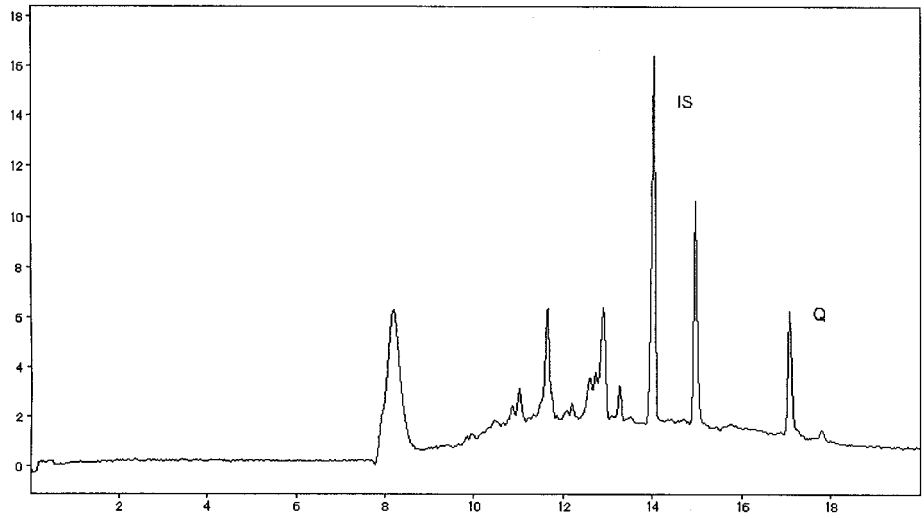

Figure 4. Electropherogram of vegetable sample (early white cabbage). Separation conditions as described in Section 2.4.

4 Concluding remarks

A method for the determination of quercetin in biological samples was developed. The method consists of hydrolysis of bonded quercetin, its isolation and preconcentration on SPE column and a final analytical step using MEKC. The working range, linear range, the limit of quantification and the measurement uncertainty were tested in validation. It was proved that the method has a sufficient robustness for the quercetin determination in plant samples.

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