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Determination of Flavonoids and Total Polyphenol Contents in Commercial Apple Juices

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Abstract

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We propose a sensitive and selective spectrofluorimetric method for the determination of flavonoids as expressed in 'quercetin equivalent' in apple juices. The method is based on the strong emission of the aluminium(III)-quercetin complex at 480 nm with excitation at 420 nm, and it is successfully applied for the determination of flavonoids in commercial apple juices and compared with results obtained in reference spectrophotometric determination. The flavonoid content in commercial apple juices was found to range from 5.53 to 15.55 mg/l quercetin equivalent. The very good agreement between the two methods indicates the suitability of the proposed spectrofluorimetric method for the precise and accurate determination of flavonoids. In addition, the total polyphenol content was determined spectrophotometrically using the Folin-Ciocalteu (FC) method and the antioxidative activity of the tested juices was tested in a DPPH assay and these values were correlated with each other. The obtained profiles of compounds with antioxidative ability lead us to conclude that fruit juice labels based only on fruit % might sometimes misinform consumers.

Keywords: aluminium; antioxidant activity; quercetin; spectrofluorimetry; spectrophotometry

Apples and apple juices are traditionally regarded as health foods all over the world. The marked antioxidant ability of phenolics from apples against free radicals is thought to account for the positive effects of apple and apple juices on human health (Hyson 2011). Numerous bioactive properties of polyphenols and flavonoids (anti-cancer, decrease of platelet aggregation, decrease of cholesterol levels and lowered risk of cardiovascular diseases) have been described (Procházková *et al*. 2011; Kay *et al*. 2012; Russo *et al.* 2012; Bubols *et al.* 2013). Polyphenolic compounds can be a major determinant of the antioxidant potential of foods (Parr & Bolwell 2000). Modifying the polyphenol content or profile could therefore bolster natural sources of antioxidants.

Apples without skin contain less than half the amount of quercetin, the most abundant apple flavonoid, harboured by whole apples (Persic *et al.* 2017). After cranberries, apples are the fruits with the second highest level of antioxidant activity. Apples also rank second in total concentration of polyphenolic compounds, and perhaps more importantly, apples have the highest proportion of free polyphenols compared to other fruits (Sun *et al.* 2002). Because the apple peels contain more antioxidant compounds than flesh, especially quercetin, apple peels may have higher antioxidant activity and higher bioactivity than the apple flesh.

Moreover, some polyphenolic compounds might be lost, and others will increase during juice production,

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packaging and storage. Apple polyphenols have been found to bind to cell wall material, which could lead to decreased levels of polyphenolic compounds in apple juices (Renard *et al.* 2001). Processing of apples has been found to affect the content of bioactive substances in apple juice (Van der Sluis *et al.* 2002). Guyot *et al.* (2003) found that only 42% of total polyphenols were extracted into the juice, leaving over half the total polyphenols in the apple pomace. However, in modern lifestyles fruit juices are consumed rather than fresh fruits. Numerous studies have focused on the effects of quercetin as a major flavonoid in apples.

Quercetin is a flavonoid derived from flavone, a natural polyphenolic antioxidant. Quercetin occurs in nature as easily hydrolysable glycoside (quercetin-3-galactoside, quercetin-3-glucoside, and quercetin-3-rhamnoside). It is known that the formation of quercetin aglycon by hydrolysis of quercetin glycosides results in an increase in activity, because the aglycon has approximately two-fold higher antioxidant activity compared to that of average quercetin glycoside (Van der Sluis *et al.* 2000).

Since quercetin is one of the most common flavonols and one of the most powerful antioxidants, it is important to have a simple, precise and accurate method for the determination of quercetin in different samples. Several methods have been proposed in the literature to determine quercetin in apple and tomato juice, fruits, wines, teas, serums and pharmaceuticals. In spectroscopic methods, the concentration of flavonoids is expressed as quercetin equivalent (QE) and quercetin is used as a standard for calibration curves. The majority of methods required some pretreatment of samples, such as solid-phase extraction (Andersen & Markham 2006).

In this study, we determined the polyphenolic compounds, flavonoids and antioxidative capacities of commercially available apple juices. We have developed a sensitive spectrofluorimetric method based on fluorescence of a complex formed between aluminium(III)-quercetin (AlQ) with flavonoids present in apple juices, and we suggest that this method is easier and more suitable in comparison to the reference spectrophotometric method.

MATERIAL AND METHODS

Reagents. All solutions were prepared in deionised water. The following chemicals were used: Folin-Ciocalteu (FC) reagent, anhydrous sodium carbonate, gallic acid, 1,1-diphenly-2-picrylhydrazyl (DPPH), quercetin-dihydrate, aluminium-nitrate and potassium acetate (all Sigma-Aldrich, USA); methanol, ethanol, sodium hydroxide and acetic acid (Merck, Germany). Acetate buffers in 70% (v/v) methanol prepared according to Perrin and Dempsey (1974) were used for all spectrofluorimetric measurements.

Instruments. The spectrophotometric measurements were performed on a Beckman DU-650 spectrophotometer, using 1 cm quartz cells.

Fluorescence spectra were collected using a Fluorolog-3 spectrofluorimeter (Jobin Yvon Horiba, France) equipped with a 450 W xenon lamp and a photomultiplier tube and using 1 cm optical path length quartz cuvettes. The slits on the excitation and emission beams were both set at 5 nm. The spectra were corrected for the dark counts. In each measurement, three scans with one-second-integration time were averaged. The emission spectrum of the solvent was subtracted. All measurements were performed at 25°C.

Measurements of pH were carried out using a Mettler Toledo MP120 pH meter (± 0.01 pH unit) equipped with a combination glass electrode.

Spectrophotometric determination of flavonoids. Quercetin was used to construct a calibration curve (standard solutions 12.5, 25, 50, 80, and 100 µg/ml in 80% ethanol (v/v)). The standard solutions or samples of juice (0.5 ml) were mixed with 1.5 ml 95% ethanol (v/v), 0.1 ml 10% $Al(NO_3)_3$, 0.1 ml of 1 mol/l potassium acetate and 2.8 ml deionised water. In the blank, the volume of 10% $Al(NO_3)_3$ was substituted by the same volume of deionised water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm. Flavonoids were expressed as QE.

Spectrofluorimetric determination of flavonoids. The stock solution of quercetin was prepared by dissolving quercetin-dihydrate in 70% methanol (v/v) and was stored in a refrigerator. A working solution (5×10^{-6} mol/l) of the AlQ complex was prepared by dilution of the stock solutions of 1×10^{-3} mol/l $Al(NO_3)_3$ and quercetin-dihydrate (1×10^{-4} mol/l).

An 0.05-ml aliquot of the tested apple juice was pipetted into a 10-ml volumetric flask, followed by 0.1 ml of $Al(NO_3)_3$, 1×10^{-5} mol/l, and the flask was filled to the top with acetate buffer pH 3.3 prepared with methanol. The fluorescence intensities of prepared solutions were measured at λ_{ex} = 420 nm and λ_{em} = 480 nm. Calibration curves obtained using

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Origin 7 software were used to calculate the quercetin content in the tested apple juices. Flavonoid levels were also expressed as QE.

The obtained data were used to calculate analytical validation parameters for both spectrofluorimetric and spectrophotometric methods according to Miller and Miller (2005) and ICH Guideline Q2B (1997).

The limit of detection (LOD) was calculated by establishing the minimum level at which quercetin can be detected, according to the followingformula:

$$LOD = 3.3\ S_b/a \quad (1)$$

where: S_b – standard deviation in intercept; a – slope of calibration line

The limit of quantification (LOQ) was determined using the following formula:

$$LOQ = 10\ S_b/a \quad (2)$$

Determination of total polyphenol content (TPC) using Folin-Ciocalteu (FC) method. Total polyphenol content (TPC) was determined spectrophotometrically according to a slightly modified FC method (Singelton & Rossi Jr. 1965; Singh *et al.* 2007). One millilitre of the sample was pipetted into a 25-ml volumetric flask containing 9 ml of water, and 1 ml of FC reagent was added. Ten millilitres of Na_2CO_3 solution were added after 5 min and the remainder of the volume was filled with deionised water. During oxidation with FC reagent phenolic compounds are reduced to blue-coloured molybdenum and tungsten oxides. After 90 min, the absorbance of the solution was measured at λ = 765 nm against a blank sample consisting of 10 ml of deionised water instead of the diluted sample. The measurements were compared to a standard curve of prepared gallic acid solutions (25–500 mg/l) and expressed as mg of gallic acid equivalents (GAE) per 1 l ± SD of apple juice. All measurements were performed in triplicate.

DPPH photometric assay. The free radical-scavenging activity of the apple juices was evaluated using the stable DPPH radical (Gardner *et al.* 2000). The hydrogen atom or electron donation abilities of the tested juices and pure quercetin were measured from the bleaching of the purple-coloured methanol solution of DPPH. One millilitre from each tested sample was added to 4 ml of 1×10^{-4} mol/l methanol solution of DPPH. After 60 min, the absorbance was recorded at 517 nm.

Inhibition of free radical by DPPH was calculated from Equation 3:

$$I(\%) = [(A_c - A_s)/A_c] \times 100 \quad (3)$$

where: A_c – absorbance of the control mixture (containing all reagents except the test compound); A_s – absorbance of the prepared sample or standard

RESULTS AND DISCUSSION

Spectrofluorometric versus spectrophotometric determination of flavonoids. A linear dependence of the absorbance of the AlQ complex (A at λ = 415 nm) on the concentration of QE (c) was observed in the interval 5–100 mg/l. For spectrophotometric determination, the calibration curve equation $A = (0.00616 \pm 0.0001)\ c + (0.00137 \pm 0.0005)$ was used. The good linearity of the calibration curve and small scatter of experimental points resulted in a high coefficient of determination, $R^2 = 0.99922$. The limit of detection (LOD) was 0.3 mg/l, while the limit of quantification (LOQ) was 0.9 mg/l.

The spectrofluorimetric determination of the content of flavonoids as QE was adapted from the procedure reported by Pavun *et al.* (2014) for quercetin determination. Spectrofluorimetric determination is based on a calibration curve ($I_F = (1.47 \pm 0.01) \times c_{Querc} + (0.56 \pm 0.04)$), where the intensity of fluorescence (%) (λ_{ex} = 420 nm and λ_{em} = 480 nm) is linearly dependent on the quercetin concentration (expressed as mg/l). A linear dependence of the fluorescence intensity of the complex was observed in the concentration range 1.5–60.5 mg/l quercetin.

The LOD was 0.09 mg/l, while the LOQ was 0.27 mg/l by the spectrofluorimetric method, much lower values than those obtained using the spectrophotometric method. The high accuracy and repeatability of the method are demonstrated by the low SD values. As we have reported in our previous work, vitamin C does not interfere with spectrofluorimetric determination of quercetin under the established experimental parameters (Pavun *et al.* 2014).

The proposed spectrofluorimetric method has a more than satisfactory sensitivity for the routine determination of flavonoid content in apple juices.

Flavonoid content in commercial apple juices. The results of the spectrofluorimetric and spectrophotometric determination of flavonoids in six apple juices available on the Serbian market are given

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Table 1. Determination of flavonoid content in commercial apple juices

Samples	Spectrophotometric	Spectrofuorimetric
	(mg/l)	
1A	5.53 ± 0.74	5.643 ± 0.004
2A	8.81 ± 0.72	8.761 ± 0.003
3A	5.43 ± 0.75	5.589 ± 0.003
1B	9.84 ± 0.80	9.772 ± 0.003
2B	15.55 ± 0.85	15.602 ± 0.004
3B	11.60 ± 0.62	11.520 ± 0.004

A – 50% of fruit nectar content; B – 100% of fruit

in Table 1. The samples were chosen from three different producers, labelled as brands 1, 2, and 3, respectively. According to the fruit juice content, samples were marked as A (50% fruit nectar) or B (100% of fruit juice).

The flavonoids content in commercial apple juices was found to range from 5.53 to 15.55 mg/l QE. The results obtained using both the spectrophotometric and spectrofluorimetric methods were in good agreement.

The amounts of bioactive compounds in fruit, including citrus flavonoids, depend on many factors, including geographic region, climate, soil properties, type of cultivar, growing season, harvest date, storage, low-dose irradiation and other conditions (Albach *et al.* 1981; Patil *et al.* 2004); therefore, the content of flavonoids as well as that of other compounds, can vary greatly.

The tested juices did not have the declared contents of flavonoids.

Determination of TPC by FC method. Polyphenolic contents of plant foods depend on a number of intrinsic (genus, species, cultivars) and extrinsic (agronomic, environmental, handling, and storage) factors (Tomás-Barberán & Espín 2001). Phenolic content in apple is also influenced by physiological disorders in fruits such as watercore and bitter pit (Zupan *et al.* 2013, 2016).

Phenolic content, which is currently considered as a measure of product quality, varied markedly according to juice processing technology and among the different brands of apple juices analysed in this study. A linear dependence of the absorbance (A at λ = 765 nm) on the concentration of GAE (c) was observed in the interval 25–500 mg/l. For the spectrophotometric determination the standard plot with equation $A = (0.00439 \pm 0.00001)^{*}c + (0.0036 \pm 0.00024)$ was used. The good linearity of the calibration curve and small scatter of experimental points resulted in a high coefficient of determination, R^2 = 0.99998. The LOD was 0.2 mg/l, while the LOQ was 0.6 mg/l. The tested apple juices did not harbour the declared contents of polyphenolics. The results obtained in this work could only be compared with already published data (Gliszczynska-Swiglo & Tyrakowska 2003) and are comparable to the available literature data concerning polyphenol content in apple juices.

DPPH photometric assay. The antioxidant activities of the commercial juices were determined using the DPPH method. All tested juices exhibited strong scavenging activity against DPPH radicals, which ranged from 32.6% to 88.26% (Table 2).

Correlation between antioxidant capacity and TPC. Numerous examples of the application of the FC assay to characterise natural products may be found in the literature. In most cases, TPC determined by this method are correlated with the antioxidant capacities, confirming the value of the FC test (Roginsky & Lissi 2005). A variety of commercial apple juices are available on the market, and TPC and antioxidant activity vary significantly among different brands. Sometimes juices labelled as consisting of 50% fruit nectar content have almost the same quality as those declared as 100% fruit juice. In this work, a high correlation was obtained between the TPC and antioxidant capacities. The TPC and the antioxidant activity both indicate product quality with respect to its biological properties, and both assays should be applied for the quality control of commercial apple juices. The TPC in commercial apple juices were found to range from 95.37 to 441.91 mg/l GAE. In order to test the correlation between the TPC and antioxidant activity of tested samples, correlation coefficients were calculated with the SPSS program

Table 2. Total polyphenol content (TPC) and inhibition activity in commercial apple juices

Samples	TPC (mg/l)	DPPH (I%)
1A	135.7	37.50
2A	191.4	85.02
3A	95.4	32.60
1B	255.9	87.85
2B	441.9	88.26
3B	264.0	86.25

A – 50% of fruit nectar content; B – 100% of fruit

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(version 18). For all brand A juices, the obtained correlation between the TPC and the antioxidant activity of tested samples was high and significant (R^2 = 0.94). For brand B juices, the obtained correlation between the TPC and the antioxidant activity of tested samples was medium-high and significant (R^2 = 0.728). The possible differences could be due to production process, apple species and the content of other compounds such as vitamin C, which may influence the antioxidant activity. Evaluation of the A and B brands together indicated a high correlation between antioxidant activity and fruit content (R^2 = 0.854).

The concentration and type of phenolic compounds varies widely in different parts of the apple fruit, such as the skin, core, seeds and the cortex (Awad *et al*. 2000). As different phenolics are present in different parts of the fruit, the concentration of phenolics in juice, puree and processed apple products may vary (Markowski & Płocharski 2006). With this in mind as well as the many other factors that may affect the antioxidant activity of commercial fruit products, it is clear that sometimes juices with less fruit nectar may have almost the same positive health benefits as juices with higher content of fruits. The antioxidative ability of fruit juices is dependent on multiple factors, and fruit juice labels stating only the fruit % can sometimes misinform the consumers, by implying, for example, that a product with a two-fold higher % of fruit is twice as good for human health.

CONCLUSIONS

In this work, we developed a simple spectrofluorimetric method for determination of quercetin in apple juice after its complexation with aluminium(III) ion. The method provides good accuracy and precision and may be used for routine analysis. Commercial apple juices were tested for TPC and antioxidative activity. The obtained profiles of compounds with antioxidative ability in commercial apple juices resulted in the conclusion that fruit juice labels based only on fruit % could sometimes misinform consumers.

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