Food and Chemical Toxicology 48 (2010) 264–270

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Determination of toxic perilla ketone, secondary plant metabolites and antioxidative capacity in five *Perilla frutescens* L. varieties

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ARTICLE INFO

Article history:
Received 27 July 2009
Accepted 5 October 2009

Keywords:
Perilla frutescens
Genotypes
Perilla ketone
Carotenoids
Phenolics
Antioxidant activity

ABSTRACT

Perilla frutescens L. is a traditional Asian crop with multiple uses. Several varieties exist but only little data is available about the content of the toxic perilla ketone and secondary plant metabolites of those genotypes. To estimate the nutritional value of this new vegetable more information about those components is necessary.

We investigated five genotypes of *P. frutescens* L. to determine their content of PK, phenolics, carotenoids and AC. AC was examined using ABTS-decolorization assay and lipid peroxidation assay. Carotenoids were identified and quantified by HPLC analysis, phenolics were quantified by means of Folin–Ciocalteu and PK was identified by GC/MS.

Two genotypes were found to contain PK, a potent lung toxin, and are therefore not suitable for consumption. The phenolic content and corresponding antioxidative capacity of all genotypes is considerably high compared to other vegetables. All genotypes moreover contain notably high concentrations of carotenoids with contents up to fivefold higher than in other carotenoid rich vegetables. The results indicate that there are several genotypes which are not suitable for consumption due to their content of PK. However PK free genotypes are rich sources of natural antioxidants, and may therefore be considered as a novel vegetable with health promoting properties.

1. Introduction

Perilla frutescens L. is a leafy vegetable widely cultivated in East Asia. Two types can be distinguished on the basis of their morphology and use: *P. frutescens* var. *frutescens* is used as an oil crop and as a common traditional vegetable in many countries in Asia. In Korea it has become one of the most important crops in recent years. The common names are Ren in Chinese, Dlggae in Korean and Egoma in Japanase. *P. frutescens* var. *crispa* is utilized for medicinal or nutritional purposes (Lee et al., 2002). The common names are Zisu in Chinese, Cha-jo-ki in Korean and Shiso in Japanese. In Korea var. *frutescens* is also used as a vegetable and the seeds are used as seasoning in China, Korea and Japan (Lee and Ohnishi, 2002). These two types of *P. frutescens* differ in morphology: var. *frutescens* is taller, has green, nonwrinkly leaves and stems and a specific fragrance. Var. *crispa* is smaller in plant height, has red or green leaves and stems and may have wrinkly or nonwrinkly leaves. Several genotypes exist of both var. *frutescens* and var. *crispa*. Up to now to our knowledge hence no data are available about the content of secondary plant metabolites and the corresponding antioxidative capacity of different perilla genotypes. In one investigation by Azuma et al. (1999) it has been shown that extracts from *Perilla ocymoides* (which is synonym for *P. frutescens*) possess high antioxidative capacity *in vitro*. In this investigation however only one genotype was included and no quantification or identification of the substances which are responsible for the antioxidative effect has been conducted. Therefore we investigated five different genotypes of *P. frutescens* L. on their content of polyphenolic compounds, carotenoids and the corresponding antioxidative capacity. Additionally we tested the five genotypes on their content of perilla ketone (PK), a potent lung toxin (Wilson et al., 1977; Tabata, 1997). Although the toxic dose of PK to humans is unknown, it is recommended not to consume PK-containing perilla species. So to introduce perilla as a novel vegetable in Europe not only knowledge about beneficial constituents like phenolics or carotenoids is necessary but is also essential to know about the PK content.

Abbreviations: ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); AC, antioxidative capacity; BHT, butylated hydroxytoluene; FW, fresh weight; GAE, gallic acid equivalents; LPO, lipid peroxidation; ORAC, oxygen radical absorbance capacity; PK, perilla ketone; TEAC, trolox equivalent antioxidant capacity.

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doi:10.1016/j.fct.2009.10.009

2. Material and methods

2.1. Chemicals

ABTS, BHT, gallic acid, trolox and carotenoid standards were obtained from Sigma Aldrich GmbH (Steinheim, Germany), Folin reagent was from Merck (Darmstadt, Germany) and POD from SERVA Electrophoresis GmbH (Heidelberg, Germany). Perilla ketone was a gift from PhD. Monica Venner, Tierärztliche Hochschule Hannover. All used chemicals and solvents were of analytical grade.

2.2. Plant material

Perilla was grown in the greenhouse under standard cultivation conditions. After 3 months leaves were harvested, freeze dried and ground to a homogenous powder. The powder was stored at −20 °C until use. Five genotypes were chosen, which differed in leaf morphology and colour:

Type 1: nonwrinkly, green leaves.
Type 2: wrinkly, green leaves.
Type 3: nonwrinkly, green, hairy leaves with purple underside.
Type 4: nonwrinkly, green, hairy leaves with purple underside.
Type 5: wrinkly, purple leaves.

2.3. Extraction and determination of perilla ketone

For extraction of perilla ketone 500 mg perilla powder were mixed with 5 ml diethylether and treated 30 min in an ultrasonic bath at 4 °C. After centrifugation the supernatant was withdrawn and analyzed by GC/MS using a Hewlett Packard GC/MS (5890 Series II) GC/MS equipped with a Supelcowax TM 10 24081 column (60m × 0.25 mm, film thickness 0.25 μm). For detection a Hewlett Packard Detector (5971 Mass selective detector) with ionization energy of 70 eV was used. Helium was used as carrier gas with a flow rate of 0.6 ml/min. Injector temperature was set at 200 °C and detector temperature at 280 °C. The temperature program was as follows: 3 min 50 °C, 10 °C/min → 120 °C, 2 min 120 °C, 3 °C/min → 136 °C, 3 min 136 °C, 9 °C/min → 240 °C, 30 min 240 °C. Sample (1 μl) was injected undiluted with the split set at 1:40. Identification of perilla ketone was based on the standard substance by comparison of retention time and mass spectrum.

2.4. Extraction of carotenoids

Carotenoids were extracted according to Hart and Scott (1995): 0.1 g perilla powder and 0.01 g potassium carbonate were mixed with 0.9 ml MeOH/THF 1:1 (v/v), thoroughly vortexed and treated 3 min in a ultrasonic bath at 4 °C. In case of extraction for HPLC analysis 0.18 ml β-apo-8′-carotenal (500 μM) was added as an internal standard and butylated hydroxytoluene (BHT) as an antioxidant. Samples for antioxidative capacity tests contained neither an internal standard nor BHT. The resulting suspension was mixed with 0.5 ml hexane and 0.5 ml 10% NaCl, again vortexed and centrifuged for 3 min (1.250 g; 4 °C). The hexane phase, which contained the carotenoids, was withdrawn. Again 0.6 ml hexane was added followed by the centrifugation step and the hexane phase withdrawn. This step was repeated another four times (altogether five extraction steps with hexane). The collected hexane phases were evaporated to dryness in a vacuum concentrator and stored at −20 °C until use.

2.5. Extraction of phenolics

For extraction of phenolics 0.5 g perilla powder was mixed with 5 ml of 80% MeOH containing 5% formic acid, thoroughly vortexed and treated in an ultrasonic bath for 60 min at 4 °C. After centrifugation (10 min, 10,000 rpm, 4 °C) the methanol-phase was withdrawn and again centrifuged (10 min, 10,000 rpm, 4 °C). Aliquots of 1 ml supernatant were evaporated to dryness in a vacuum concentrator and stored at −20 °C until use.

2.6. Analysis of carotenoids

Carotenoids were resolved in 500 μl CH_2Cl_2 and 1500 μl EtOH, diluted 1:2 with EtOH and analyzed by HPLC according to Sander et al. (1994) using a Merck Hitachi L 6200 A intelligent pump with a Phenomenex Develosil RP aqueous C30-column (250 × 46 mm, 5 μm) and an UV–Vis Detector (Merck Hitachi L-4250). Peaks were quantified with software from Merck Hitachi (D-7000 HPLC System Manager 4.1). The gradient consisted of MeOH + 0.05% triethylamine (A), acetone (B) and H_2O + 0.05 M ammoniumacetate (C) as solvents (Table 1). Injection volume was 20 μl, column oven was set at 25 °C, detection wavelength at 450 nm, flow rate was 1 ml/min. For each genotype two independent extracts were analyzed each two times ($n = 4$) (see Fig. 1).

2.7. Quantification of phenolics

Phenol extract was resolved in 500 μl 80% MeOH containing 5% formic acid, diluted 1:100 with ethanol and reducing agents were quantified by means of Folin–Ciocalteu (Singleton et al., 1998). Results are given as gallic acid equivalents (GAE). For each genotype two independent extracts were analyzed each three times ($n = 6$).

2.8. Antioxidative capacity in the ABTS-system

For detection of antioxidative capacity (AC) a 2,2′-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) decoloration assay was used (Cano et al., 2000). The assay contained in a total volume of 2 ml: 1 mM ABTS, 6 μM peroxidase (POD), 0.7% acidified EtOH (0.35 ml ortho phosphoric acid in 50 ml EtOH), water and EtOH. All reagents were solved in ethanol, with exception of POD, which was solved in H_2O. The ABTS radical cation was generated by adding 35 μM H_2O_2. Carotenoid samples were solved in 500 μl CH_2Cl_2 and 500 μl EtOH and diluted 1:5 with EtOH. Phenols were resolved in 500 μl 80% MeOH containing 5% formic acid and diluted 1:100 with EtOH. The absorption of the radical was determined at 734 nm (UV–Vis Photometer Pharmacia LKB Biochrom 4060). After 2 min 100 μl of sample were added and after 6 min absorption was determined again. The absorption difference is a measure for AC. AC is given as trolox equivalent antioxidative capacity (TEAC). For each genotype two independent extracts were analyzed each three times ($n = 6$).

2.9. Antioxidative effects against of lipid peroxidation (LPO)

Kinetics of lipid peroxidation (LPO) was measured in plasma according to Schnitzer et al. (1998). Plasma was diluted 1:5 in PBS. Carotenoid extracts were resolved in 500 μl CH_2Cl_2 and 1500 μl EtOH, phenolic extracts were resolved in 500 μl

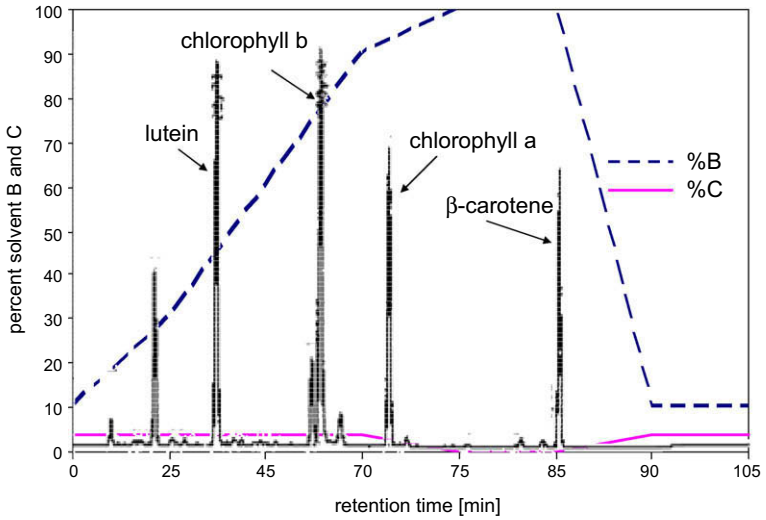

Fig. 1. Elution profile of carotenoids.