EVALUATION OF FLAVONOIDS AND FUROCOUMARINS IN BERGAMOT DERIVATIVES BY HPLC-DAD

VANESSA GIANNETTI (*)1 - MAURIZIO BOCCACCI MARIANI (*) - ELENA TESTANI (*) - VIRGINIA D'AIUTO (*)

Abstract

The high concentration of bergamot polyphenolic fraction is generating growing interest in all its derivatives due to the potential beneficial effects on human health, suggesting new application in the functional foods field. Once widely used in perfumery for the exclusive fragrance of its essential oil, the bergamot has reported, in the last decades, a drop in demand due to the synthetic essence placing on the market. In order to re-launch this citrus on the international market, the dry juice extract seems to be particularly suitable to be mixed with other foods with minimal impact on their organoleptic properties.

The characterization of bergamot derivatives (juice, pastazzo and dry juice extract) was performed with HPLC-DAD method, with the detection and quantification of some flavonoids such as neohesperidin, naringin e neoeriocitrin, which represent an antioxidant pattern almost unique in nature, and of two furo-coumarins, bergapten and bergamottin, potentially toxic if beyond certain limits. The method was validated in terms of detection limits (LODs), linearity, precision and accuracy.

Riassunto

L'elevata concentrazione della frazione polifenolica del bergamotto sta generando crescente interesse verso tutti i prodotti da esso derivati per i potenziali

(*) Department for Technologies, Resources and Development, Sapienza University of Rome, Via del Castro Laurenziano 9, 00161, Rome, Italy.
1 Corresponding author: vanessa.giannetti@uniroma1.it

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effetti benefici sulla salute umana, suggerendo nuove applicazioni nel campo degli alimenti funzionali. Un tempo molto impiegato in profumeria per la fragranza esclusiva del suo olio essenziale, il bergamotto ha registrato negli ultimi decenni un notevole calo di richiesta dovuto all'immissione sul mercato dell'essenza sintetica. Nell'ottica di un rilancio di tale agrume sul mercato internazionale in campo alimentare, l'estratto di succo essiccato si presta perfettamente ad essere miscelato con altri alimenti con impatto minimo sulle loro proprietà organolettiche. La caratterizzazione dei derivati del bergamotto è stata condotta mediante metodica HPLC-DAD, con l'individuazione e la quantificazione di alcuni flavonoidi, tra cui neoesperidina, naringina e neoeriocitrina, che rappresentano un pattern antiossidante pressoché unico in natura, e di due furocumarine, bergaptene e bergamottina, potenzialmente tossiche se presenti oltre certi limiti. Il metodo è stato validato in termini di LODs, linearità, precisione ed accuratezza.

Keywords: Bergamot, bergamot dry juice extract, flavonoids, furocoumarins, HPLC-DAD.

Introduction

Bergamot (*Citrus Bergamia Risso*) is a fruit produced almost exclusively in the region of Calabria (Italy). This citrus is considering by many to be a natural hybrid of bitter orange and lemon. Bergamot plants grow in a narrow coastal strip of about 150 km, in the area of Reggio Calabria, which presents particular favourable weather and pedoclimatic conditions for its cultivation. The cultivated area is of about 1,450 hectares with an annual production of 140,000 fruit tons.

Up to now this fruit has been mainly used for the extraction of the essential oil from the peel; the most profitable product of the bergamot industrial processing. The bergamot essential oil possesses a very intense and exclusive fragrance due to the abundant presence of terpens, esters, and alcohols, and it is mainly used by the cosmetic industry since it can fix the perfume aromatic bouquet. The medicinal properties of bergamot are known since antiquity; for a long period the bergamot essence has been used by the pharmaceutical industry for its antiseptic and antibacterial characteristics so as to be inserted in the pharmacopoeia of several countries. Recent studies have shown how some bergamot characteristics are particularly interesting in supporting the metabolic pathologies treatment such as dyslipidemia and metabolic syndrome which are correlated with cardiovascular pathologies and atherosclerosis (1-2).

Moreover, the traditional medicine highlights the relaxing properties of the bergamot extracts and the important nutraceutical properties of the fruit. The bergamot essence is also used by food and confectionery industry as liquors, teas and sweets aroma. However, in the last decades, the advent of the synthetic reconstruction of the bouquet of bergamot oil has led to a dramatic decrease in demand.

In the last years, bergamot has attracted considerable attention for its high antioxidant content, since, like all citrus fruits, it is characterized by a substantial amount of flavonoids. Their profile in the fruits depends on the different species and cultivar, the degree of ripeness and the pedoclimatic environment, and it presents a typical pattern in the various parts of the same fruit. The composition of the bergamot polyphenolic fraction, flavanone component mainly, is about 100 times richer than in the other Citrus species (orange, lemon) (3-9). The presence of these polyphenolic compounds with their health-related properties could lead to a complete and rational use of bergamot (10-11).

Recent studies have showed that the richness in such compounds makes the bergamot derivatives very interesting under the nutritional profile, suggesting application in nutraceutic field. However, nowadays, bergamot juice obtained from the endocarp after essential oils extraction is considered just a secondary or discarded product in the working diagram of this citrus fruit (12). On these grounds it seemed interesting to investigate all possible uses of the juice in order to take advantage from the large amount of this discarded product.

Beside the significant polyphenolic component that can be used as phytocomplex for anticholesterolemic therapeutic formulations in food field, the bergamot fruit has a polysaccharide fraction usable as base for food integrators and a fibrous-woody fraction that can be used in diet products in order to reduce hunger sense.

The main products derived from bergamot fruit are the juice, hard to market for its strong organoleptic properties that cause an unpleasant taste; and the *pastazzo* powder particularly rich in polyphenols (until 3-5 times more than in the juice) that could be used for infusions, herb-tea, decoctions or to be mixed with other food products with a minimal impact on their organoleptics properties (yogurt, cereals, fruit juices). From the bergamot juice and *pastazzo* we can get a dry extract that, once made hydro-soluble, can be use in drops, syrups or creams.

The literature data show that the main bergamot antioxidant components are three flavanones (neohesperidin, naringin, neoeriocitrin) that represent a characteristic pattern almost unique in nature (13). Moreover, the presence of other flavonoids promise possible new applications of these citrus in food field in order to add value to a natural product still considered as a discarded product.

The use in food field has been limited by the discovery of the potentially toxic action of some bergamot compounds, such as furocoumarins (in particular the bergapten) which require removal procedures for a safety use. Linear furocoumarins (psoralens) are widely found in plants but are particularly abundant in the Umbelliferae/Apiaceae and Rutaceae.

They can be dangerous to humans, since they can cause photosensitization toward UV light (resulting in sunburn or serious blistering) and promote skin pigmentation when they are administrated topically (14-17).

The aim of this study was to develop a HPLC-DAD procedure allowing the qualitative and quantitative evaluation of flavonoids and furocoumarins in the different parts of the bergamot fruit.

Experimental

Chemicals

Methanol for HPLC was purchased from Merck (Darmstadt, Germany), acetic acid and formic acid from Carlo Erba, ultrapure water was obtained by Milli-Q water system (Millipore, France). Neoeriocitrin, naringin, neohesperidin, bergapten and bergamottin (purity > 98%) were from Sigma-Aldrich (Milano, Italia). Bergamot fruits came from Reggio Calabria territory.

Sample treatment, extraction procedure, analytical determination

Bergamot fruits were collected in January 2009 in a plantation located near Reggio Calabria. The fresh fruits were exhaustively washed and treated by specific procedure. Once bergamot derivatives were obtained, they were stored in 20 mL aliquots in glass vials and immediately frozen at −20 °C. The juice samples were prepared using a manual citrus-fruit squeezer; the peel samples were removed from the fresh fruit, the *pastazzo* samples were obtained by homogenization of the peeled fruit after squeezing and the lyophilized juice extract was purchased in capsules available on the market and used as received.

In order to determine the flavonoidic component and the furocoumarins in the different bergamot products a solvent-extraction procedure was optimized. In the case of *pastazzo* and peel, 5 mg of sample was accurately weighed into a glass stoppered vials; for the juice 10 mL were measured. The extraction solvent was a solution of 1% formic acid in methanol (18). The extraction solvent was added to the sample in the ratio 1:4 (v/v for the juice and w/v for *pastazzo*, peel and lyophilised material), shaken briefly, sonicated in ultrasonic bath and centrifuged for 10 min at 1000 rpm. The resulting organic solution was filtered through filter paper (Millipore 0.22 µm) in order to remove solid residues and it was injected into the HPLC system.

The two furocoumarins (bergapten and bergamottin) were separated and simultaneously quantified with the flavonoidic compounds. The determination of the analytes was carried out by liquid chromatography, employing a Waters (mod.600, Milford, MA) HPLC system equipped with a Waters diode array detector (mod.2998) and using the Empower 2 Software for the signal acquisition and elaboration. A 20 µL sample volume was used for the HPLC analysis, carried out by a Symmetry® C18 column (75 × 4.6 mm i.d., 3.5 µm), operating at 1.0 mL/min. The mobile phase was methanol and 5% (v/v) acetic acid aqueous solution. The gradient was: 5-20% (0-13 min), 20-100% (13-25 min), 100-5% (20-30 min).

Detection was performed by monitoring the absorbance signals between 210-350 nm and the data elaboration was carried out selecting the wavelength of maximum absorption of the analytes: i.e. 283 nm. Flavonoids and furocoumarins contained in the extracts were identified by comparing their retention times and UV spectra to external standards and by the sample enrichment.

Validation procedure

In order to develop an analytical method suitable for quantitative determination of flavonoids and furocoumarin traces in bergamot fruits, linearity, detection limits (LOD), precision and accuracy were determined. Quantitative analysis of each compound was carried out by comparing the peak areas in the sample with those of the external standard solutions. The latter were prepared in methanol in the concentration range of 8-100 mg/L and kept at 4 °C, in dark condition, until their use. Calibration curves for each analytes were plotted in order to determine the linearity range and the detection limits.

The analysis were conducted in triplicate and the quantification was achieved by external standard calibration. Calibration curves for all analytes showed excellent linear response, in the considered range, as confirmed by the correlation coefficients ($r^2 > 0.9982$). The detection limits (LODs) were calculated as the concentration corresponding to the lowest signal above baseline for a signal/noise ratio of 3:1. By this criterion, the LOD values were ranging between 0.01 and 0.02 mg/mL. The precision of the analytical method was evaluated by performing ten repetitive injections of a diluted standard solution. The relative standard deviations (RSDs) of the peak areas were found to be from to 0.8 to 3.9% showing a good precision. In order to evaluate the method accuracy, recoveries were assessed by comparing the concentration in spiked bergamot juice samples with known amounts of each standard compound and by performing quadruplicate assays before and after addition. The average recoveries were between 91.1 and 97.3%.

Results and discussion

The presence and the abundance of a wide variety of flavonoids in bergamot fruits, essential for their antioxidant power, promises a possible future applications of these substances in the food field. The aim of this work was to develop a simple analytical procedure for the determination of polyphenolic compounds in bergamot fruits and their furocoumarin content, whose toxicity represents a limit for a bergamot safe employment in the food field. The optimized method was applied to bergamot juice, *pastazzo* and peel samples in order to evaluate flavonoids and furocoumarins distribution in the fruit components. The obtained data showed significant amounts of neohesperidin, naringin and neoeriocitrin in all parts of the bergamot fruit. The presence of this pattern is almost unique in nature and, as a consequence, these compounds could be used as markers in order to detect possible fraudulent juices adulteration (19).

The bar chart of Figure 1 shows a remarkable difference in the distribution of the flavonoidic portion and of the furocoumarins in bergamot juice, *pastazzo* and peel. The analysis of sample extracts highlighted a greater distribution of the flavonoidic component in peel and *pastazzo* than in juice and an appreciable amount of bergamottin in peel in respect of the juice. In particular, a five times greater content of neohesperidin, naringin, neoeriocitrin and bergamottin is achieved in peel extract than in the fruit juice. Whereas, the greater bergapten content in peel than in juice is not thus remarkable as the other analytes.

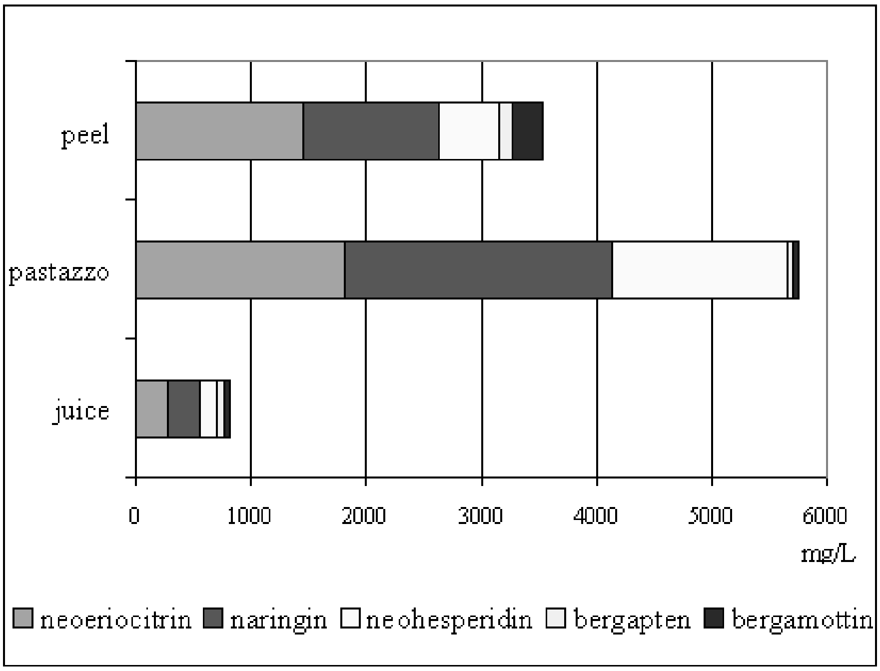

Fig.1 - Distribution of flavonoids and furocoumarins in bergamot juice, pastazzo and peel.

The chromatographic profile, reported in Figure 2, shows a great amount of the flavonoidic component in bergamot juice, justifying the growing interest in this citrus in the field of functional food.

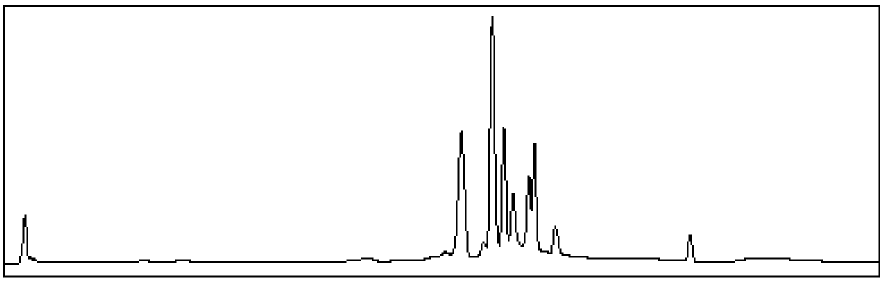

Fig. 2 - Chromatographic profile of bergamot juice at 283 nm; 1. neoeriocitrin, 2. naringin, 3. neohesperidin, 4. bergapten, 5. bergamottin (chromatographic conditions in Experimental part.

Dry extract of the bergamot juice, a powder product available on the market, was then analyzed to evaluate the flavonoidic component and the furocoumarins content in order to assess a possible use in the food field. The powder was contained in 500 mg capsules. A comparison between the weight percentages of each compounds in bergamot juice and in the dry extract is reported in Table 1.

TABLE 1

FLAVONOIDS AND FUROCOUMARINS CONTENT IN BERGAMOT JUICE AND DRY EXTRACT SAMPLES (%w/w)

Component	Bergamot juice	Dry extract in capsules
Neoeriocitrin	0.028	3.680
Naringin	0.028	15.12
Neohesperidin	0.014	12.68
Bergapten	0.007	0.269
Bergamottin	0.005	n.d.

n.d.: not detected

The reported data shows an high level of the typical flavonoidic profile and a lower furocoumarin content in the dry extract due, probably, to the process of capsules preparation.

This data can be considered meaningful for a future re-launch of bergamot on the functional food market. In effects, the criticalities linked to the use of the bergamot juice in the food field, such as its physical characteristics (it is fermentable, therefore needs to be maintained on the cold line) and its organoleptic properties (no appreciable taste and, consequently, it is difficult to put it on the market), could be overcome with the use of dry extract which is rather easily storable and lends itself being mixed with other foods with minimal impact on their organoleptic characteristics.

In conclusion, the proposed method, based on the use of a C_{18} column and a linear HPLC gradient, proved to be simple, efficient and rapid, and it can be considered as a valuable instrument for routine analysis for polyphenolic substances determination in food matrix.

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REFERENCES

(1) N. MICELI, M.R. MONDELLO, M.T. MONFORTE, V. SDRAFKAKIS, P. DUGO, M.L. CRUPI, M.F. TAVIANO, R. DE PASQUALE, A. TROVATO, "Hypolipidemic effects of *Citrus bergamia Risso* et Poiteau juice in rats fed a hypercholesterolemic diet", *J. Agric. Food Chem. 2007*, 55, 10671-10677.

(2) P.M. KRIS-ETHERTON, K.D. HECKER, A. BONANOME, S.M. COVAL, A.E. BINKOSKI, K.F. HILPERT, T.D. ETHERTON, "Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer", *Am. J. ed. 2002*, 113, 71-88.

(3) G. GATTUSO, D. BARRECA, C. GARGIULLI, U. LEUZZI, C. CARISTI, "Flavonoid composition of Citrus juices", *Molecules 2007*, 12, 1641-1673.

(4) Y. NOGATA, K. SAGAMOTO, H. SHIRATSUCHI, T. ISHII, M. YANO, H. OHTA, "Flavonoid composition of fruit tissues of citrus species", *Biosci. Biotechnol. Biochem. 2006*, 70, 178-192.

(5) G. GATTUSO, C. CARISTI, C. GARGIULLI, E. BELLOCCO, G. TOSCANO, U. LEUZZI, "Flavonoid glycosides in bergamot juice (Citrus bergamia Risso)", *J. Agric. Food Chem. 2006*, 54, 3929-3935.

(6) A. BOCCO, M.E.CUVELIER, H. RICHARD, C. BERSET, "Antioxidant activity and phenolic composition of Citrus peel and seed extracts", *J. of Agric. and Food Chem. 1998*, 46, 2123-2129.

(7) K. ROBARDS, X. LI, M. ANTOLOVICH, S. BOYD, "Characterization of citrus by chromatographic analysis of flavonoids" *J. of the Sci. of Food and Agric. 1997*, 75, 87-101.

(8) S. KAWAII, Y. TOMONO, E. KATASE, K. OGAWA, M. YANO, "Quantitation of flavonoid constituents in citrus fruits", *J. Agric. Food Chem. 1999*, 47, 3565-3571.

(9) P. SWATSITANG, G. TUCKER, K. ROBARDS, D. JARDINE, "Isolation and identification of phenolic compounds in citrus sinensis", *Anal. Chim. Acta 2000*, 417, 231-240.

(10) O. BENAVENTE-GARCÍA, J. CASTILLO, F.R. MARIN, A. ORTUNO, J.A. DEL RIO, "Uses and properties of citrus flavonoide", *J .Agric. Food Chem. 1997*, 45(12), 4505-4515.

(11) C. KAUR, H.C. KAPOOR, "Antioxidants in fruits and vegetables-the millennium's health", *Int. J. Food Sci. Technol. 2001*, 36, 703-725.

(12) A. DI GIACOMO, "Il Bergamotto di Reggio Calabria", *Laruffa Editor 1989*, 102-119.

(13) W. OOGHE, "Flavonoids as authenticity markers for Citrus sinensis juice", *Fruit Process 1999*, 8, 308-313.

(14) S. KAWAII, Y. TOMONO, E. KATASE, K. OGAWA, M. YANO, "Isolation of furocoumarins from bergamot fruits as HL-60 differentiation-inducing compounds", *J. Agric. Food Chem. 1999*, 47, 4073-4078.

(15) G. EISENBRAND, "Toxicological assessment of furocoumarins in foodstuffs", *Mol. Nutr. Food Res. 2007*, 51, 367-373.

(16) A. BOWERS, "Phytophotodermatitis", *Am. J. Contact Derm. 1999*, 10, 89-93.

(17) B. GIRENNAVAR, S.M. POULOSE, G.K. JAYAPRAKASHA, N.G. BHAT, B.S. PATIL, "Furocoumarins from grapefruit juice and their effect on human CYP 3A4 and CYP 1B1 isoenzymes", *Bioorg. Med. Chem. 2006*, 14, 2606-2612.

(18) C. GARDANA, F. NALIN, P. SIMONETTI, "Evaluation of flavoniods and furanocoumarins from Citrus Bergamia (Bergamot) juice and identification of new compounds", *Molecules 2008*, 13, 2220-2228.

(19) D. CAUTELA, B. LARATTA, F. SANTELLI, A. TRIFIRÒ, L. SERVILLO, D. CASTALDO, "Estimating bergamot juice adulteration of lemon juice by High-Performance Liquid Chromatography (HPLC) analysis of flavanone glycosides", *J. Agric. Food Chem. 2008*, 56(13), 5407-5414.