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Food Chemistry 87 (2004) 537–541
Food Chemistry
www.elsevier.com/locate/foodchem

Free and conjugated polyamine content in *Citrus sinensis* Osbeck, cultivar Brasiliano N.L. 92, a Navel orange, at different maturation stages

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Received 20 October 2003; accepted 7 January 2004

Abstract

Biogenic amines, synthesized during physiological metabolic processes of all living organisms, are present in food. At low concentrations, polyamines are essential for cell renewal and growth, but they can be detrimental when consumed in high amount through the diet as they could support abnormal cell growth pathologies. The daily human diet contains more putrescine than spermidine or spermine, mostly derived from fruits. In general, orange fruits contain high levels of put, a fact that could limit their utilization in the daily diet besides the benefits contributed by their strong antioxidant properties. There is therefore an increasing interest in finding plant foods with low polyamine contents, which could provide a staple diet for patients. This paper reports the amounts of free and conjugated polyamines in the flesh and peel (flavedo plus albedo) of unripe, ripe and over ripe fruits of the cultivar Brasiliano, a Navel group orange. The analyses reveal that this particular orange cultivar has low polyamine content and could be suitable for a low polyamine diet.

Keywords: Polyamines; Orange; Spermidine; Putrescine; Conjugated polyamines

1. Introduction

Natural amines are nitrogenous compounds in which alkyl groups replace 1, 2 or 3 hydrogen atoms in ammonia and they can be classified as monoamines, diamines and polyamines according to the number of basic groups (NH or NH_2) present in their molecules (Bagni & Tassoni, 2001).

Diamines and polyamines are widely distributed in animals, plants and microrganisms and have been associated with many different processes. In fact, putrescine (put), spermidine (spd) and spermine (spm) may act as growth factors, are involved in every step of DNA, RNA and protein synthesis and are essential for cell proliferation and growth (Bagni & Tassoni, 2001). Since most of the key genes involved in the polyamine biosynthetic pathway in plants have been recently cloned and sequenced, it has been possible to manipulate polyamine biosynthesis using antisense and sense transgenic approaches, with the aim of increasing or depleting the total polyamine content (Burtin & Michael, 1997; Pedros et al., 1999; Thu-Hang, Bassie, Safwat, Trung-Nghia, Christou, & Capell, 2002).

In all living organisms, biogenic amines are synthesized during physiological metabolic processes (Bardócz, Grant, Brown, Ralph, & Pusztai, 1993) and are therefore present in food. The levels of amines in plant food products may vary according to the ripening degree, storage and growth conditions, variety of plant and possible bacterial contaminations. Biogenic amines can

Abbreviations: cad: cadaverine; dap: 1,3-diaminopropane; HPLC: high performance chromatography; PCA: perchloric acid; put: putrescine; spd: spermidine; spm: spermine.

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doi:10.1016/j.foodchem.2004.01.001

also be formed during product storage or processing by thermal or bacterial enzymatic decarboxylation of free amino acids (Halász, Baráth, Simon-Sarkadi, & Holzapfel, 1994; Mulas, Gonzales-Aquilar, Lafuente, & Zacarias, 1998).

The daily human diet contains more put than spd or spm, mostly derived from cheese and non-green vegetables, in particular potatoes and fruits (Bardócz et al., 1995). At low concentrations, polyamines are essential for cell renewal and growth, but they can be detrimental when consumed in high amounts through the diet because they could support, together with other growth factors, abnormal cell growth such as cancer pathologies (Bardócz et al., 1999; Pryme, Tiburcio, Flores, & Mattsson, 1998). The inhibition of polyamine biosynthesis in cancerous tissues is a major target for scientists involved in polyamine research and efficient blockers of in vitro cell proliferation have been developed even if they are not always effective in vivo (Mc Cann, Pegg, & Sjoerdsma, 1987). For this purpose it is essential to determine the polyamine concentration in different components of the diet in order to limit the amounts of polyamines daily ingested with the food. There is in fact an increasing demand for plant foods (cereals, potatoes, fruits and vegetables) with low polyamine contents, which can provide a staple diet for patients suffering for cancer or other forms of unwanted cell growth (Bardócz et al., 1999).

Studies on the influence of free polyamines during development of fruits, such as avocado, mandarin, apple, grape, litchi and olive, revealed peak levels in put and spd during the early stages and then decreasing values as the fruit becomes completely ripe (Kakkar & Ray, 1993; Shiozaki, Ogata, & Horiuchi, 2000). Smith (1982) has reported that polyamines, naturally present in vegetables, play an important role in cell division regulation and in plant growth, and found that the maximum level of put coincides with the expansion of fruit weight and volume. In tomatoes, the high put levels and the rapid cell proliferation that occurs during the early stages of fruit growth, suggest an influence of polyamines on cell division (Kakkar & Ray, 1993; Teitel, Cohen, Arad, Burnbaum, & Mizrahi, 1985).

Regarding orange fruit, which plays a very important role in the Mediterranean diet, the possibility of limiting polyamine content, as well as utilizing a variety with low endogenous polyamine levels, is relevant, even taking into consideration the benefit bestowed by the strong antioxidant properties of this fruit. It was in fact reported that orange fruits and juices contain more put than other fruits and vegetables (Bardócz et al., 1999; Okamoto, Sugi, Koizumi, Yanagida, & Udaka, 1997), a fact that could limit their use in the daily diet of some particular pathogenic individuals.

This paper reports the amounts of free and conjugated (to hydroxycinnamic acids) polyamines in the flesh and peel (flavedo plus albedo) of unripe, ripe and over ripe fruits of the cultivar Brasialiano N.L. 92, an orange of the Navel group.

2. Materials and methods

2.1. Plant material

Brasiliano N.L. 92 is a sweet orange cultivar (*Citrus sinesis* Osbeck) of the Navel group, selected among nucellar seedlings obtained from the cultivar Brasiliano, widely grown in the western part of Sicily. The distinctive feature of this kind of oranges is the presence of a small secondary fruit (navel) in the apex of the primary fruit. For this and other reasons (bitterness of the stored juice), they are mainly used for the fresh fruit market and not for processing (Hodgon, 1967). Regarding the morphological characteristics of the trees, they are vigorous, large, with dark green and thick leaves, with sterile flowers (sterile pollen and rare viable ovules) and a good and constant productivity. Fruits are medium-large (about 190 g in weight), early-medium ripening (December–January), seedless, with a navel evident in the 20% of cases.

Fruits are well accepted from the market because of their characteristics of easy peeling and separation of segments, seedless, excellent flavour and deep orange colour of the rind and of the juice. Ripe fruits are resistant on the tree and they have a long shelf-life.

The analyses were carried out on fruit samples collected from cultivar Brasiliano N.L. 92, cultivated at the experimental orchard of the Istituto di Genetica Vegetale C.N.R. in Lascari (Palermo, 38°N). All plants, each 25 years old, are grafted on *Citrus aurantinum* L. The morphological observations were carried out at three different periods of the year: the beginning of October (unripe fruits) end of November (ripe fruits) and the end of February (over ripe fruits). For each data point four perfectly healthy, representative fruits were picked from three different trees, at the four cardinal points and from the external part of the canopy. The fruits were saved in dry ice for the first 24 h and at −20 °C until analyses.

2.2. Polyamine analysis by HPLC

The analyses were carried out separately on flesh and peel (flavedo plus albedo) orange tissues. The samples were collected from mixed tissues derived from four fruits from three different trees, and the data represent the means of two separate determinations performed in triplicates.

Polyamine analyses were performed according to Tassoni, van Buuren, Franceschetti, Fornalè, and Bagni (2000). Orange flesh (about 40 gFW) and peel (about 20 gFW) samples were homogenised in 10 v of 4% (v/v) cold perchloric acid (PCA) and centrifuged at 20,000*g* for 30 min at 4 °C. The pellet was washed three times and resuspended in the original volume of PCA. Triplicates of this suspension and of the supernatant were hydrolysed with 6 N HCl in flame-sealed vials at 110 °C for 20 h in order to release polyamines from their conjugates. Aliquots (0.2 ml) of supernatant (free polyamines), hydrolysed supernatant (PCA-soluble conjugated polyamines) and hydrolysed pellet (PCA-insoluble conjugated polyamines) were derivatised with dansyl–chloride (3 $mg\,ml^{-1}$ of acetone), toluene-extracted and analysed by HPLC (Jasco, Großumstad, Germany) with a reverse phase C_{18} column (Spherisorb ODS2, 5 μM particle diameter, 4.6 × 250 mm), as described by Tassoni et al. (2000). Standard polyamines were subjected to the same procedure.

3. Results and discussion

Polyamines in the free, PCA-soluble and PCA-insoluble conjugated form, were present in Brasiliano N.L. 92 orange tissues, both in the flesh and in the peel (flavedo plus albedo). Free put was the most abundant polyamine, both in the flesh and peel, followed by free spd, while free spm was present only in traces, this confirming previous findings (Bardócz et al., 1993; Eliassen, Reistad, Risoen, & Ronning, 2002; Okamoto et al., 1997). Free 1,3-diaminopropane (dap) was prevalently present in ripe fruits (Fig. 1).

The amount of free put increased, both in the peel and in the flesh during ripening, going from unripe to ripe fruits, and decreased in over ripe oranges, reaching maximum values in the peel (1093 nmol gFW^{-1}) and in the flesh (493 nmol gFW^{-1}) of ripe fruits. A similar trend, but with lower amplitude, was also shown for spd (Fig. 1).

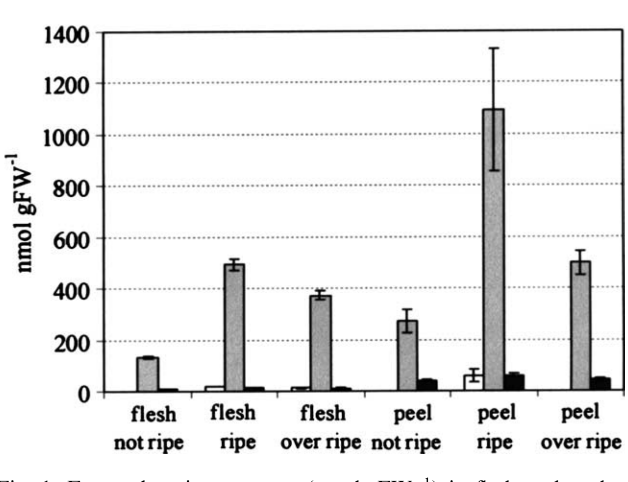

Fig. 1. Free polyamine contents (nmol gFW^{-1}) in flesh and peel of Brasiliano N.L. 92 orange fruits. (□), dap; (▒) put; (■) spd. Values represent the means ± SE of three determinations.

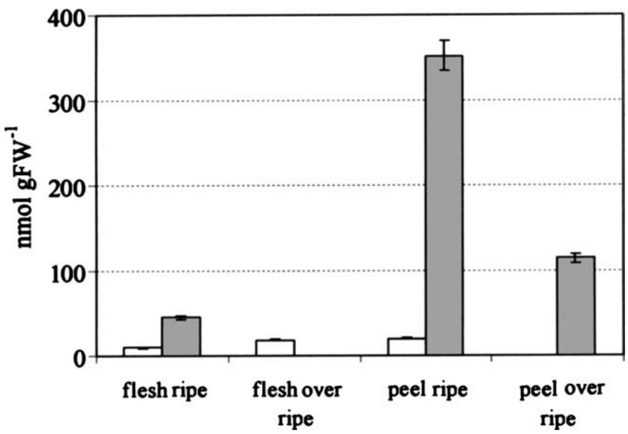

Fig. 2. PCA-soluble conjugated polyamine contents (nmol gFW^{-1}) in flesh and peel of Brasiliano N.L. 92 orange fruits. (□), dap; (▒) put. Values represent the means ± SE of three determinations.

PCA-soluble conjugated polyamines (covalently bound to monomers of hydroxycinnamic acids) were detected only in ripe and over ripe orange fruits (Fig. 2). Putrescine was the most abundant PCA-soluble conjugated polyamine, reaching a maximum in the peel of ripe fruits, dap was present at low concentration, and spd and spm were not detectable in any analysed sample.

Polyamines present in the PCA-insoluble fraction are generally conjugated with dimers of hydroxycinnamic components or branched cinnamic acids that also bind the cell wall components, such as hemicelluloses (Bagni et al., 2000). PCA-insoluble conjugated polyamines seemed to have the same trend of free polyamines, even if at about 100-fold lower concentration. Conjugated put in the PCA-insoluble fraction occurred in all the analysed samples, being particularly abundant in the peel of ripe fruits. Spermidine and, for the first time, cadaverine (cad) covalently bound in the PCA-insoluble fraction, were only detectable in unripe and ripe fruits (Fig. 3). Spermine and dap were not present in the PCA-insoluble fraction.

Polyamines conjugated to PCA-soluble and PCA-insoluble fractions represented about 20% of the total polyamine amount present in the peel of ripe and over ripe fruits and, respectively, 20% and 6% in the flesh of ripe and over ripe oranges. Putrescine conjugated with hydroxycinnamic acids in the PCA-soluble fraction of orange fruits was previously identified by Wheaton and Stewart (1965) as feruloylputrescine.

Taking into consideration the free, PCA-soluble, PCA-insoluble and total polyamine contents of Brasiliano N.L. 92, it was possible to confirm that Brasiliano N.L. 92 orange fruits contain less total polyamines (592 nmol gFW^{-1}) than other orange cultivars previously analysed (Table 1) such as Tarocco and Valencia (Bagni et al., 2000). Total free polyamine amount of Brasiliano N.L. 92 fruits seems to be considerably lower than other

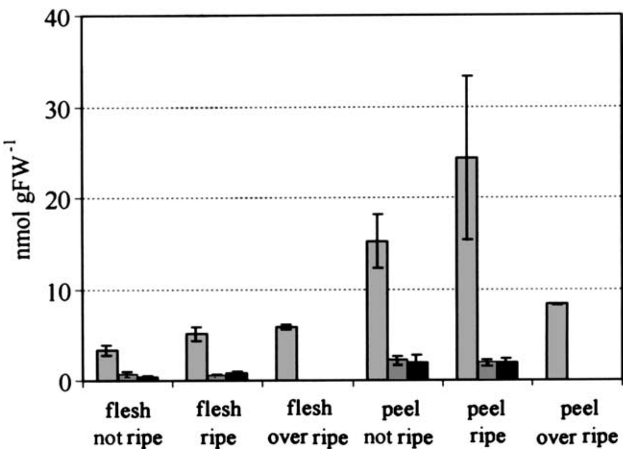

Fig. 3. PCA-insoluble conjugated polyamine contents (nmol gFW^{-1}) in flesh and peel of Brasiliano N.L. 92 orange fruits. (■), put; (■) cad; (■) spd. Values represent the means ± SE of three determinations.

Table 1
Polyamine levels (nmol gFW^{-1}) in orange fruits of different cultivars

Orange cultivar	Polyamine levels (nmol gFW^{-1})			Data source
	Free	Conjugated	Total	
Brasiliano N.L. 92	529	62.6	591	This paper
Valencia	9905	573	1562	Bagni et al. (2000)
Tarocco	439	345	784	Bagni et al. (2000)
Not specified cultivar	1351	N.d.	–	Okamoto et al. (1997)
Not specified cultivar	1585	N.d.	–	Eliassen et al. (2002)
Not specified cultivar	1142–1646	N.d.	–	Bardócz et al. (1993)
Orange juice	312–348	N.d.	–	Bardócz et al. (1993)

N.d., not determined.

reported orange free polyamine levels and of the same order of magnitude as Tarocco which, on the contrary, contains a higher amount of conjugated polyamines (Table 1) (Bardócz et al., 1993; Eliassen et al., 2002; Okamoto et al., 1997). Moreover, the free polyamine levels of Brasiliano N.L. 92 fruits seem to be of the same magnitude order as the reported orange juice levels (Bardócz et al., 1993) and comparable to kiwi (60.7 ± 27.9 nmol gFW^{-1}), a fruit very rich in vitamin C and antioxidants (Bagni et al., 2000).

Well known is the role, in human health, of free polyamines, administered with the food at low and high concentrations, while very little information is available on the role of polyamines conjugated to hydroxycinnamic acids. Pharmacological investigations about feruloylputrescine and naturally occurring amides (Wheaton & Stewart, 1965), indicated that these compounds have hypotensive activity and, in addition, amides are scavengers of free radicals (Bors, Langebartels, Michel, & Sandermann, 1989).

In conclusion, this study shows that the cultivar Brasiliano N.L. 92, an orange of the Navel group, has a lower polyamine content than other cultivars previously tested and it could be considered for a diet with low daily polyamine content.

Acknowledgements

This research was supported by the funds of FIRB project "Sicurezza ed aspetti tecnico-economici e giuridici delle produzioni biologiche", financed by Ministry of University, Science and Technological Research of Italy to Nello Bagni and by the funds of the MiPAF Programme "Ricerche e Sperimentazioni nel Settore dell'Agrumicoltura Italiana" to Maria Antonietta Germanà, Paper No. 54.

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