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Non-fluorescent and yellow chlorophyll catabolites in Japanese plum fruits (*Prunus salicina*, Lindl.)

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ABSTRACT

Although several chlorophyll metabolites have been shown to exert prominent benefits to human health when consumed, the battery of linear chlorophyll derivatives (phyllobilins) presents in fruits is poorly understood. Yellow chlorophyll catabolites (YCCs) are a new kind of phyllobilins recently identified in senescent leaves, probably arising from an oxidative process of the terminal chlorophyll catabolites, NCCs (non-fluorescent chlorophyll catabolites). This work deals with the characterization by first time of this kind of phytochemicals in edible fruits. Two YCCs have been identified in yellow Japanese plums, one (*Ps*-YCC1) previously described in the senescent leaves of *Cercidiphyllum japonicum* Siebold & Zucc. and *Ps*-YCC2, a chlorophyll catabolite structure described by first time in the edible parts of Japanese plum fruits. These YCCs were characterized by high-resolution MS/MS, describing the specific fragmentation (ring A) and the absence of the typical cleavage of phyllobilins (ring D), as a consequence of the unsaturated bond at C15–16 typical of YCCs, allowing the differentiation from NCCs. To the already known array of phenolic acids, anthocyanins and carotenoids, NCCs and YCCs may contribute to the antioxidant potential of these fruits, a potential that deserves attention and future research, considering the photochemical and photophysical behaviour of this group of tetrapyrrolic breakdown products.

1. Introduction

Chlorophylls are vital pigments in all photosynthetic tissues, including the non-foliar ones like the fruits where photosynthesis contributes to their own growth (Blanke & Lenz, 1989; Sánchez & Harwood, 2002). The chlorophyll pigments are catabolized during the ripening of fruits and senescence of leaves, generating a variety of chlorophyll catabolites (Kräutler, 2014). Although chlorophyll catabolites have been considered just the final products of chlorophyll catabolism without any further function in the plant tissue, it has recently been shown that structurally related chlorophyll derivatives may exert prominent benefits to human health when consumed (Roca, Chen, & Pérez-Gálvez, 2016), benefits derived from different biological actions like antioxidant (Ulbricht et al., 2014), antimutagenic (Ferruzzi, Böhm, Courtney, & Schwartz, 2002) and antigenotoxic (Jonker et al., 2002). The first part of the chlorophyll degradation pathway takes place in the chloroplast through modifications of the cyclic tetrapyrrolic derivatives (chlorophyllide, pheophytin and pheophorbide). Subsequently, PaO (pheophorbide *a* oxygenase; Pružinska, Tanner, Anders, Roca, & Hörtensteiner, 2003) enzyme opens the characteristic macrocycle of pheophorbide *a*, yielding the linear chlorophyll catabolites denominated phyllobilins. The first phyllobilin structure originated is the RCC (Red Chlorophyll Catabolite) that quickly is transformed into a *p*FCC (primary fluorescent chlorophyll catabolite; 1-formyl-19-oxobilin or phyllobilins type I) by RCC reductase (Wüthrich, Bovet, Hunziker, Donnison, & Hörtensteiner, 2000). *p*FCC is exported from the chloroplast to the cytosol where several enzymes introduce specific functional groups in restricted peripheral positions of its structure. Thus, only three positions are subjected to modifications, the C18 (R_1 in Table 1) can be dihydroxylated or not, the $C8^2$ (R_3 in Table 1) can be methyl esterified or not, and the $C3^2$ (R_2 in Table 1) can be hydroxylated, and subsequently bonded to specific substituents. Additionally, the FCCs can be deformylated at C1 originating the DFCCs (1,19 dioxobilins or phyllobilins type II; Christ et al., 2013). Finally, modified FCCs/DFCCs are transported into the vacuole where they are isomerized to the final NCCs/DNCCs (non-fluorescent chlorophyll catabolites) by a non-enzymatic reaction (Oberhuber, Berghold, Breuker, Hörtensteiner, & Kräutler, 2003). As mentioned before, NCCs/DNCCs have been historically considered the final catabolite products of the chlorophyll degradation pathway (Kräutler, 2014), but still they may

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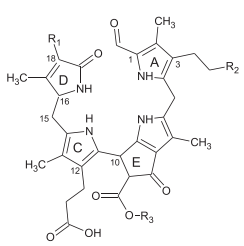
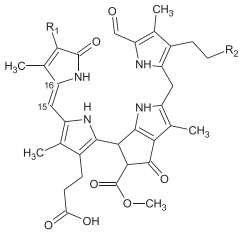
Table 1
Structures of YCCs previously identified and phyllobilins characterized in *Prunus salicina*, Lindl.





Compound	R_1	R_2	R_3
YCCs			
Cj-YCC1	$CH=CH_2$	OH	
Tc-YCC1	$CH(OH)-CH_2OH$	O-β-glucopyranosyl	
Ed-YCC1	$CH(OH)-CH_2OH$	3-Oxo-propionic	
Phyllobilins in *Prunus salicina*			
Ps-NCC1	$CH=CH_2$	OH	H
Ps-NCC2	$CH=CH_2$	O-β-glucopyranosyl	CH_3
Ps-YCC1	$CH=CH_2$	OH	
Ps-NCC3	$CH=CH_2$	OH	CH_3
Ps-YCC2	$CH=CH_2$	O-β-glucopyranosyl	

Cj: *Cercidiphyllum japonicum*, *Tc*: *Tilia cordata*, *Ed*: *Egeria densa*; *Ps*: *Prunus salicina*.

develop some remarkable actions. In fact, NCCs from fruits have been found to be effective antioxidants (Müller, Ulrich, Ongania, & Kräutler, 2007), so that new hypotheses about possible physiological roles of these catabolites in the senescent tissue have emerged like their role to inhibit the decline of vital functions during the ripening of fruits. The number of characterized NCCs/DNCCs to date is 17 (Pérez-Gálvez & Roca, 2017), mainly in senescent leaves but also in some fruits like apples and pears (Müller et al., 2007), loquats (Ríos, Roca, & Pérez-Gálvez, 2014b), quince (Ríos, Pérez-Gálvez, & Roca, 2014) and lemons (Ríos, Roca, & Pérez-Gálvez, 2015). The nomenclature system for phyllobilins (Ginsburg & Matile, 1993) consists on the initials of the Latin name of the species where the phyllobilin is described, followed by the abbreviation of the phyllobilin (FCC, NCC, DNCC, *etc*.) with a number that indicates the polarity. Consequently, the same phyllobilin structure could present different names depending on the vegetal species where it has been identified.

It is documented that NCCs are easily oxidized; in fact, they were firstly named as rusty pigments (Kräutler, Jaun, Bortlik, Schellenberg, & Matile, 1991). Surprisingly, a yellow chlorophyll catabolite (YCC) was described in the senescent leaves of *Cercidiphyllum japonicum* and proposed to be an endogenous oxidative derivative from the *Cj*-NCC1 (Table 1) by a simple dehydrogenation at the C15–16 bond (Moser, Ulrich, Müller, & Kräutler, 2008). This yellow chlorophyll catabolite exhibited an UV–vis spectrum with two characteristic maxima, at 310 nm and 426 nm. The first maximum, also showed by the type-I phyllobilins, is due the α-formyl group at C1, and the exclusive maximum at 426 nm is consistent with the presence of the double bond between the C/D rings. Recently, several YCCs have been described in the senescent leaves of the Lime tree (*Tilia cordata*) (Scherl, Müller, & Kräutler, 2012) and the senescent leaves of *Egeria densa* Planch. (Wakana et al., 2014) and tentatively identified in leaves of *Prunus domestica* L. (Erhart et al., 2016) and *Prunus armeniaca* L. (Mittelberger et al., 2017). The YCCs are structurally derived from known NCCs structures (Table 1), and although *Cj*-YCC has been chemically synthesized by oxidation of *Cj*-NCC1, the enzymes and processes involved the biochemical origin of these yellow chlorophyll catabolites remains elusive.

The present work describes the identification of a new YCC by first time in a ripened fruit, the Japanese plum fruits of the yellow cultivar *Prunus salicina* L. In addition to the novelty of the characterization of a new phyllobilin, the results obtained confirm that the chlorophyll degradation pathway is highly similar in fruits and leaves, a hypothesis sometimes questioned (Banala et al., 2010).

2. Materials and methods

2.1. Plant material

Yellow fruits of the Japanese plum tree (*Prunus salicina*, Lindl., variety Golden Japan) were purchased in a local market. Maize (*Zea mays* L.) seeds were germinated to harvest primary leaves, while leaves of spinach (*Spinacia oleracea* L.) were bought at a local market. They were allowed to senescence by dark-incubation in distilled water in Petri dishes at 25 °C during 5 to 7 days (Matile, Ginsburg, Schellenberg, & Thomas, 1988). NCCs present in the extracts from senescent leaves of maize and spinach were used as standards for characterization of the chlorophyll catabolite profile in Japanese plum fruits.

2.2. Reagents

Potassium phosphate was provided by Sigma-Aldrich Chemical Co. (Madrid, Spain). HPLC LC/MS grade solvents were supplied by Panreac (Barcelona, Spain). The deionized water used was obtained from a Milli-Q 50 system (Millipore Corp., Milford, MA, USA). Sodium formate (NaCOOH) (10 mM NaOH in 300 μL of formic acid) was used for calibration.

2.3. Non-fluorescent chlorophyll catabolites extraction

Fresh material (20 g of peels from 10 Japanese plum fruits, and 20 g of senescent leaves of spinach and maize) was homogenized in liquid nitrogen and extracted into 10 volumes of ice-cold acetone during 30 min in a vortex at maximum speed at 4 °C. The extract was centrifuged at 14,000 × *g* for 5 min and the pellet was extracted again. Supernatants from both extractions were joined and concentrated in a rotary evaporator. The aqueous residue was partially diluted with ice-cold MeOH (20%) and applied to a SPE column (C_{18}, Bakerbond SPE, 500 mg/6 mL, J.T. Baker, Deventer, Holland) activated with two volumes of methanol and two volumes of water. The SPE with the sample was cleaned with four volumes of water for desalting, and the non-fluorescent chlorophyll catabolites fraction was eluted with 1 mL of 20 mM potassium phosphate pH 7.0/methanol (1:9, v/v) (Mühlecker & Kräutler, 1996; Roca, 2012). The sample was stored at − 20 °C until analysis. The experimental procedures were made under diminished light.

2.4. Liquid chromatography/electrospray ionization/time-of-flight mass spectrometry

The liquid chromatograph system was Dionex Ultimate3000RS U-HPLC (Thermo Fisher Scientific, Waltham, MA, USA). Chromatographic separation was performed as described earlier (Berghold, Breuker, Oberhuber, Hörtensteiner, & Kräutler, 2002) but with modifications detailed by Ríos, Pérez-Gálvez, and Roca (2014) and Ríos, Roca, and Pérez-Gálvez (2014). A split post-column of 0.4 mL/min was introduced directly on the mass spectrometer electrospray ion source. The HPLC/ESI-QqTOF operated for mass analysis using a micrOTOF-QII High Resolution Time-of-Flight mass spectrometer (UHR-TOF) with Qq-TOF geometry (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization (ESI) source. The acquisition of the MS spectra was performed as described by Ríos et al. (2015). Mass spectra were acquired in MS fullscan mode and data were used to perform multi-target-screening using TargetAnalysis™ 1.2 software (Bruker Daltonics, Bremen, Germany). MS^2 spectra were acquired in Auto-MS^2 mode