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Citrus hydrosols as useful by-products for tyrosinase inhibition

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ABSTRACT

Hydrosols are coproduced during water or steam distillation of plant material. Three kinds of citrus hydrosols (CIHSs) were assessed for anti-tyrosinase (TYR) activity using spectrophotometric assays and terpene content as quantified by gas chromatography (GC). All of the distillate waters were found to inhibit commercial mushroom tyrosinase at varying levels (21.8–68.9%) depending on substrate type and concentration. The GC analysis indicated that a number of known tyrosinase inhibitors including myrcene, sabinene, geraniol and citral were present in CIHS, which behave as mixed-type inhibitors towards tyrosinase.

Industrial relevance: Citrus hydrosols have great potential to meet the demands of the food and cosmetic industries, since they are not only easy and inexpensive to produce but also without any perceivable hazard for humans. In addition, since citrus hydrosols can be extracted from the discarded peels of citrus fruits, their use as anti-browning agents would allow the repurposing of what has typically been considered a biological waste product.

1. Introduction

Hydrosols are by-products coproduced during water or steam distillation of plant material. Since they contain trace amounts of essential oils as well as other hydrophilic dissolved compounds, hydrosols are commonly used for aromatherapeutic and cosmetic purposes (Inouye, Takahashi, & Abe, 2008). Moreover, several authors (Lin, Yang, Wu, Kwan, & Chen, 2011; Sagdic, 2003; Tajkarimi, Ibrahim, & Cliver, 2010; Tornuk, Ozturk, Sagdic, Yilmaz, & Erkmen, 2014; Tornuk et al., 2011) showed the possibility of using herbs and spice hydrosols in drinks, food preservation and as a convenient sanitizing agent during the washing of fresh-cut fruits and vegetables. When considering the use of hydrosols for food applications, a very important characteristic is that they are considered free from side effects for humans, as is the case for essential oils, which are listed in the Code of Federal Regulation as generally recognized as safe (GRAS) (FDA, 2013; Kabara, 1991).

In particular, citrus oils, which are the focus of this study, are mainly used for the flavouring of fruit beverages, confectioneries, and soft drinks, as well as for the perfuming of eau de cologne, soaps, cosmetics and household products (Raeissi, Diaz, Espinosa, Peters, & Brignole, 2008). They are also employed in medical treatments and are known to have antimicrobial properties, including antifungal, antibacterial, antiviral and antiparasitic activities (Rehman et al., 2007). Citrus peel essential oils are contained in oil sacs, or vesicles, located in the outer rind or flavedo of the fruit. The peel oil is a by-product of citrus juice extraction usually recovered by mechanical separation, known as cold-pressing, hydrodistillation or steam distillation (Lota, De Rocca Serra, Tomi, Jacquemond, & Casanova, 2002). Distillation is an economical way to recover the oils, with a better yield (0.21%) than cold pressing (0.05%) (Ferhat, Meklati, & Chemat, 2007). Moreover Sahraoui, Vian, El Maataoui, Boutekedjiret, and Chemat (2011) extracted essential oil from orange peels with microwave steam distillation in comparison to the conventional steam distillation. Results confirm the effectiveness of this technique which allows the reduction of time and energy of extraction without causing changes in the volatile oil composition.

During distillation, citrus peels are exposed to boiling water or steam to release their essential oils through evaporation. As steam and essential oil vapours condense, both are collected and separated in a vessel, and hydrosols are recovered and usually discarded.

Citrus is the most abundant fruit crop in the world (about 131 millions tons in 2012 (FAOSTAT, 2013)) and the amount of waste obtained from citrus fruits accounts for 50% of the whole fruit (Braddock, 1995; Chon & Chon, 1997). As reported by Sahraoui et al. (2011) transformation of citrus wastes allows balancing their processing cost with value added output and environmental protection. To our knowledge, no study demonstrated yet the ability of citrus hydrosols to control the enzymatic browning that may be considered a bridge between the food and cosmetic fields. On the basis of these considerations, citrus hydrosols could have great potential for further commercial use because TYR (EC 1.14.18.1) is the main enzyme responsible for the browning reaction of fruits and vegetables, and it is involved in the initial reaction of

Abbreviations: CH, citron hydrosol; CIHS, citrus hydrosol; GC, gas chromatography; L-DOPA, L-3,4-dihydroxyphenylalanine; LH, lemon hydrosol; OH, orange hydrosol; *t*-BC, *tert*-butylcatechol; TYR, tyrosinase.

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melanin pigment synthesis (Kim & Uyama, 2005). This enzymatic reaction can lead to alteration of colour and a partial loss of the antioxidant capacity of some foods due to the generation of more *o*-quinone and melanisation in animals. The most frequently used inhibitors of enzymatic browning in industry include ascorbic acid and various forms of sulfite-containing compounds. The latter have applications for a broad range of products and are strong anti-browning and antimicrobial agents Fan, Sokorai, Liao, Cooke, and Zhang (2009). However, adverse side effects such as high toxicity towards cells and low stability when exposed to oxygen and water limit their application (Schurink, van Berkel, Wichers, & Boeriu, 2007). Recently, TYR inhibitors from natural sources have became popular as food and cosmetic additives to prevent enzymatic browning (Loizzo, Tundis, & Menichini, 2012; Parvez, Kang, Chung, & Bae, 2007).

As reported by Zocca, Lomolino, and Lante (2011), dog rose and pomegranate extracts obtained with minimal processing can be used as anti-browning agents to preserve the quality of fresh-cut vegetables and fruit. In addition, products enriched with bioactive compounds such as those present in dog rose hips and pomegranate may prove to be an effective tool to both develop functional foods and to increase the overall intake of plant products. Exploiting the promise of the wastewater agri-food industry, Zocca, Lomolino, and Lante (2010) suggested that also *Brassicacea* processing water, that is a source of bioactive compounds, may be useful for the control of enzymatic browning throughout a given product/service lifecycle.

The effectiveness of natural products is mainly attributed to their high content of bioactive components as organic acids, glucosinolates and polyphenols. In particular, polyphenols represent a diverse group of compounds containing multiple phenolic functionalities and identified as specific inhibitors of TYR (Chang, 2009). Furthermore polyphenols have been recognized as having many health benefits mainly due to their antioxidant activity (Kang, Shin, Lee, & Lee, 2011; Lante & Friso, 2013; Lante, Nardi, Zocca, Giacomini, & Corich, 2011; Mihaylova, Lante, Tinello, & Krastanov, 2014).

The purpose of the present work is to investigate possible new uses as anti-browning agents for three different citrus hydrosols that are usually discarded even if may contribute to extra business profit as natural additives, providing a connection between the needs of improve quality and reduce chemical substances in the food and cosmetic fields.

2. Material and methods

2.1. Reagents

Commercial mushroom tyrosinase (EC 1.14.18.1), L-3,4-dihydroxyphenylalanine (L-DOPA), (−)-epicatechin, *tert*-butylcatechol (*t*-BC), hexane, acetone, n-dodecane and GC standards myrcene, α-terpinene, (R)-limonene, terpinolene, sabinene, α-terpineol, geraniol, citral (mixture of *cis* and *trans* isomers, ≥96%), and β-citronellol were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Citrons (*Citrus medica* (L.)), lemons (*Citrus limon* (L.) Burm. cv. "Femminello"), and oranges (*Citrus sinensis* (L.) Osbeck cv. "Tarocco") were purchased at commercial maturity from a local store. All citrus fruits were produced organically.

2.2. Sample preparation

Fruit was washed under running water to remove any surface contamination and wiped with blotting paper. After that fruit was peeled so as to separate the inner layer (albedo) from the outer one (flavedo). This fraction, rich in oil glands, was cut into small pieces (about 0.5 cm), crushed in liquid nitrogen, weighed and distilled. For laboratory-scale experiments, citrons, oranges and lemon peels (100 g) were distilled by adding 300 ml of distilled water with Cazenave equipment consisting of a steam generator, a flask with steam pipe, a distillation column and a condenser.

Two distillations were done for each fruit. Distillates (250 ml) were collected and analysed to determine terpene content and TYR inhibition capacity in the CIHS. After steam distillation, the oleous phase was completely separated from water by centrifugation at 4 °C at 12,000 rpm for 5 min. Citron hydrosol (CH), lemon hydrosol (LH), orange hydrosol (OH) were kept in air-tight sealed glass vials, covered with aluminium foil at 4 °C until further analysis.

2.3. TYR activity inhibition

Commercial TYR was dissolved in 0.1 M sodium citrate buffer at pH 6.0 to a final concentration of 1336 U/ml. TYR activity was assayed spectrophotometrically at 475 and 440 nm with 10 mM L-DOPA or (−)-epicatechin respectively in a sodium citrate buffer. The solution used for a blank included 1.0 ml of L-DOPA or (−)-epicatechin at different concentrations, 300 μl of distilled water and 10 μl of commercial TYR (added last). Sample reaction mixtures were obtained by substituting 300 μl of inhibitors (CH, LH, and OH) with distilled water. Absorbances at 440 nm and 475 nm were monitored at 25 °C using a UV/Vis spectrophotometer (JASCO 7800, Tokyo, Japan). Only the linear part of the curve (Δ absorbance vs time) was taken into account to calculate enzyme activity.

The percent inhibition of TYR activity was calculated as follows (Baurin, Arnoult, Scior, Do, & Bernard, 2002):

$$\%\text{TYR inhibition} = \left[\left(A_{control} - A_{sample}\right)/A_{control}\right] \times 100\% \quad (1)$$

where $A_{control}$ = absorbance at 440 nm and 475 nm without test sample and A_{sample} = absorbance at 440 nm and 475 nm with test sample. L-Ascorbic acid was used as a positive control. Enzyme activity was calculated in the linear part of the curve after the lag phase (Alam et al., 2011). The assay was carried out in air-saturated aqueous solutions. Inhibitory kinetics of samples were analysed using Lineweaver–Burk plots. The kinetic data were plotted as 1/activity ($1/V_0$) versus 1/substrate concentration ($1/S$), according to the method of Lineweaver–Burk, and the Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) were determined with variable substrate concentrations in the standard reaction mixture.

2.4. Gas chromatography (GC) analysis

Terpene quantification was performed by liquid–liquid extraction and GC. To determine terpene content of CIHS, a GC 8000 Top CE Instruments (Thermo Finnigan) gas chromatograph equipped with a flame ionization detector (FID) and AS 2000 auto injection sampler was used. Hydrogen was used as a carrier gas with a flow rate of 22.4 cm/s and flux of 0.37 ml/min at 297 Pa. The column was a DB-1 (40 m × 0.1 mm I.D. and 0.2 μm film thickness; Agilent J&W, USA). Injector and detector temperatures were 250 °C. The oven temperature program was as follows.

Rate (°C/min)	Iso T (°C)	Iso t (min)
–	–	1
–	60	1
4	250	10

CIHS samples (9 ml) were transferred to test tubes containing 3 ml of hexane (volume ratio of 3:1). They were sealed with rubber caps and agitated using a vortex mixer for 1 min. After phase separation, 0.950 ml of hexane was transferred to tubes containing the internal standard (50 μl), and 1 μl of this solution was injected into a gas chromatograph. As an internal standard, n-dodecane dissolved in acetone (100 mg/100 ml) was used. Identification of terpenes was