

SUPPLEMENTARY MATERIAL

Characterization of the polyphenolic fraction of pomegranate samples by comprehensive two-dimensional liquid chromatography coupled to mass spectrometry detection

Marina Russo¹ Francesco Cacciola^{2*}, Katia Arena³, Domenica Mangraviti³, Laura de Gara¹, Paola Dugo^{1,3,4}, Luigi Mondello^{1,3,4}

¹Department of Medicine, University Campus Bio-Medico of Rome, via Álvaro del Portillo 21, 00128 Rome

²Dipartimento di Scienze Biomediche, Odontoiatriche e delle Immagini Morfologiche e Funzionali, University of Messina, Via Consolare Valeria, 98125 Messina, Italy

³Dipartimento di Scienze Chimiche, Biologiche, Farmaceutiche ed Ambientali, University of Messina, Polo Annunziata, Viale Annunziata, 98168 Messina Italy

⁴Chromaleont s.r.l., c/o Dipartimento di Scienze Chimiche, Biologiche, Farmaceutiche ed Ambientali, University of Messina, Polo Annunziata, Viale Annunziata, 98168 Messina, Italy.

*Corresponding author. Prof. Francesco Cacciola Tel. +39 0906766570.

E-mail address: cacciolaf@unime.it

Abstract

Punica granatum L., commonly known as pomegranate, is an ancient fruit widely consumed all over the world as fresh fruit or juice. In addition, it is extensively used in therapeutic formulas, cosmetics and food seasonings. The fruit is native to Afghanistan, Iran, China and the Indian sub-continent. The pomegranate market has steadily grown, presumably due to the increasing demand of health-conscious consumers for products with potential beneficial effects on human health, due to the synergistic presence of a unique and complex phytochemical composition that enclose anthocyanins, phenolic acids and hydrolysable tannins. Conventionally, for their analysis liquid chromatography is employed even though it can present some drawbacks in terms of resolving power. In this contribution, as a valuable alternative, comprehensive two-dimensional liquid chromatography with “shifted gradients” in the second dimension, was applied for the characterization of three pomegranate samples, leading to the identification of 37 different polyphenolic compounds.

Keywords: *Punica granatum* L.; polyphenols; comprehensive two-dimensional liquid chromatography; mass spectrometry.

Experimental section

Reagents and plant material

All following solvents were purchased from Sigma-Aldrich, (Milan, Italy): Water, acetonitrile, methanol and formic acid LC-MS grade.

Pomegranate fruits (Wonderful cultivar), collected from Italy, were purchased at a local market. Voucher specimens are deposited at the Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, under accession number n° 23/17.

Sample and sample preparation

Pomegranate fruits were harvest in 2017 when commercially ripe, after 15 weeks from flower set, and in according to external changing colour. The arils were manually separated from the pomegranate fruit of about 644 g. The skins, once recovered, were cut into strips and dried for 12 hours at 30° C, they were then shredded by a coffee grinder. The powder (2.5 g) was added twice with 25 mL of acidified water at 0.1% of formic acid. The residue thus obtained was sonicated for 15 minutes, then centrifuged (3.000 × g, for 10 min.). Finally, the aqueous fraction was evaporated with a rotary evaporator and added 25 mL of ethanol to extract the most apolar polyphenols. The

dried extract was resolubilized with acidified water at 0.1% of formic acid and filtered through a 0.45 μm Acrodisc nylon membrane (Merck Life Science, Merck KGaA, Darmstadt, Germany) prior to LC and LC \times LC injection.

The pulp (2.5 g) was added twice with 25 mL of acidified water at 0.1% of formic acid. Afterwards it was sonicated for 15 minutes, and then centrifugated (3.000 \times g, for 10 min.) to separate the juice. The remaining fraction was dried by rotary evaporation and the dried extract was extracted again with acidified water. At the end, the evaporated fraction was recovered with acidified water at 0.1% of formic acid and filtered through a 0.45 μm Acrodisc nylon membrane prior to the LC and LC \times LC analysis. The juice obtained from pulp centrifugation was injected as it was in the LC and LC \times LC system.

Instrumentation and Software

LC and LC \times LC analyses were performed on a Nexera-e liquid chromatograph (Shimadzu, Kyoto, Japan), consisting of a CBM-20A controller, four LC-30AD dual-plunger parallel-flow pumps, a DGU-20A₅R degasser, a CTO-20AC column oven, a SIL-30AC autosampler, an SPD-M30A photo diode array (PDA) detector (1.0 μL detector flow cell volume). The two dimensions were connected by means of two high speed/high pressure two-position, six-ports switching valves with micro-electric actuator (model FCV-32 AH, 1.034 bar; Shimadzu, Kyoto, Japan), placed inside the column oven and equipped with two 20 μL stainless steel loops. The Nexera-e liquid chromatograph was hyphenated to an LCMS-8050 mass spectrometer, through an ESI source (Shimadzu, Kyoto, Japan). The LC \times LC-MS system and the switching valves were controlled by the Shimadzu Labsolution software (ver. 5.65). The LC \times LC data were visualized and elaborated into two and three dimensions using Chromsquare ver. 2.3 software (Shimadzu, Kyoto, Japan).

Analytical conditions

LC separations

LC separations were carried out on an Ascentis Express C18 column (Merck Life Science, Merck KGaA, Darmstadt, Germany, 150 \times 4.6 mm I.D., 2.7 μm d.p.). The mobile phases were: (A) 10% formic acid in water, (B) water-ACN-formic acid (40:50:10 v/v/v) ; Gradient: 0 min, 12% B; 35 min, 30% B; 36 min, 100% B (held for 4 min); 40 min, 12%B; flow rate: 1 mL/min. Oven: 30°C. Injection volume: 5 μL .

LC \times LC separations

¹D separations were carried out on an microbore Ascentis ES-Cyano column (Merck Life Science, Merck KGaA, Darmstadt, Germany, 150 \times 1.0 mm I.D., 2.7 μm d.p.). The mobile phases were: (A) 0.1% formic acid in water (pH 3), (B) 0.1% formic acid in ACN ; Gradient: 0 min, 5% B; 30 min,

10% B; 100 min, 25% B (held for 20 min); 160 min, 100%B; flow rate: 0.015 mL/min. Oven: 30°C.
Injection volume: 5 μ L;

2 D separations were carried out on an Ascentis Express C18 column (Merck Life Science, Merck KGaA, Darmstadt, Germany, 50 \times 2.1 mm I.D., 2.0 μ m d.p.). The mobile phases employed were (A) 0.1% formic acid in water (pH 3), (B) 0.1% formic acid in ACN. Shift gradient (SG) conditions as reported in Figure S2. The flow rate employed was 0.8 mL min $^{-1}$. Modulation time of the switching valves: 1.00 min. Loop internal volume: 20 μ L. Column oven: 30°C.

Figure S1. RP-LC plot ($\lambda=280$ nm) of detected polyphenolic compounds in pomegranate juice

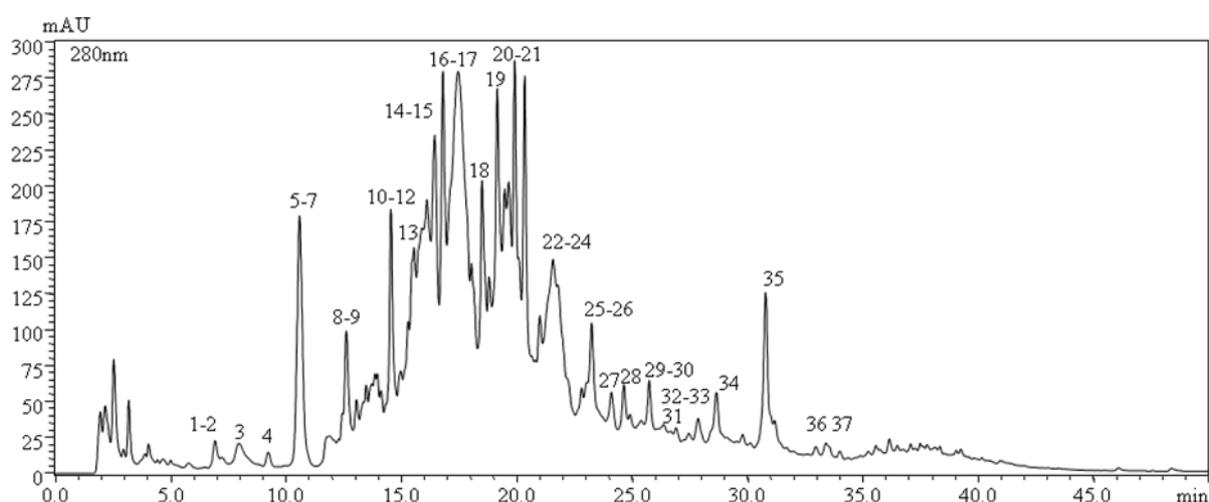


Figure S2. Shift gradient in 2 D analysis

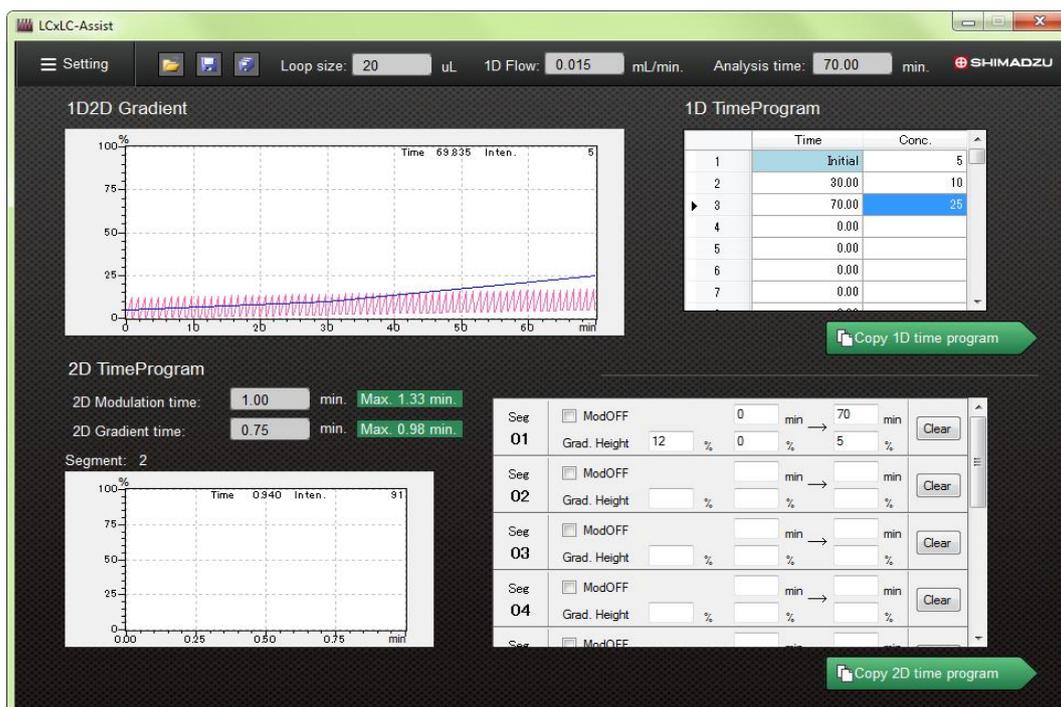


Figure S3. RP-LC×RP-LC plot ($\lambda=280$ nm) of detected polyphenolic compounds in pomegranate juice

