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Oxyprenylated Ferulic Acid Derivatives in Italian Citrus Liqueurs

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Abstract

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4'-Geranyloxyferulic and boropinic acid have been recently found as phytochemicals exerting promising pharmacological effects as cancer chemopreventive, anti-inflammatory, neuroprotective, and anti-*Helicobacter pylori* agents. An RP HPLC-UV/Vis method for the qualitative and quantitative analysis of these oxyprenylated derivatives in handmade citrus-based typical Italian liqueurs like limoncello, arancello, and mandarinetto was successfully performed. Concentration values showed a great variation between the different liquors, limoncello, from Abruzzo region, being the richest one both in 4'-geranyloxyferulic acid (1.68 ± 0.03 µg/ml of liquor) and boropinic acid (0.16 ± 0.01 µg/ml). Only in two cases boropinic acid was not detected.

Keywords: boropinic acid; *Citrus* spp.; 4'-geranyloxyferulic acid; limoncello; arancello; mandarinetto; Rutaceae

Citrus fruits are often used as ingredients of several food preparations, like marmalades, candied peels, and beverages. Among them are the typical Italian liqueurs obtained by maceration of citrus fruits like lemons, oranges, and mandarins in a saccharose hydroalcoholic solution providing the spirit drinks locally known as limoncello, arancello, and mandarinetto, respectively. All are greatly appreciated for their aroma and taste, as well as for their digestive properties, once consumed after dinner, and are also used as ingredients for the preparation of several cocktails due to their strong flavours deprived of the sourness and bitterness of pure lemon, orange, or mandarin juices. Analysis of phytochemicals from limoncello has been the topic of several papers recently published in the literature revealing that this liqueur contained volatiles (terpenes, alcohols, and aldehydes) (Versari *et al*. 2003), phenylpropanoids (Dugo *et al*. 2003), and several other secondary metabolites (Locatelli *et al*. 2012). On the other hand, less has been reported on the chemical composition of arancello and mandarinetto, for which, to the best of our knowledge, only three studies have been published in the literature reporting the presence of volatiles (Schipilliti *et al*. 2013). Resulting from an ethnically ancient tradition and being used as digestives in several parts of the world research on the chemical composition of Italian citrus-based liqueurs is a field of current interest.

Oxyprenylated natural compounds, i.e. 3,3-dimethylallyloxy-(isopentenyl, C_5), geranyloxy- (C_{10}), and farnesyloxy- (C_{15}) related compounds, represent a group of rarely occurring secondary metabolites. These phytochemicals have been found in the last two decades as valuable products exerting promising and interesting pharmacological effects (Epifano *et al*. 2007; Genovese *et al*. 2014). We have recently found that citrus fruits can be seen as valuable sources of oxyprenylated ferulic acid derivatives like 3-(4'-geranyloxy-3'-methoxyphenyl)-2-*trans* propenoic acid ((*E*)-3-{4-[(2*E*)-3,7-dimethylocta-2,6-dienoxy]-3-methoxy-phenyl}prop-2-enoic acid according to IUPAC nomenclature), more commonly known as 4'-geranyloxyferulic acid 1 (herein designated with the acronym GOFA) and boropinic acid 2 ((*E*)-3-[3-methoxy-4-(3-methylbut-2-enyl)oxy)phenyl]prop-2-enoic acid according to IUPAC nomenclature) (herein designated with the acronym BPA), the structures of which are illustrated in Figure 1.

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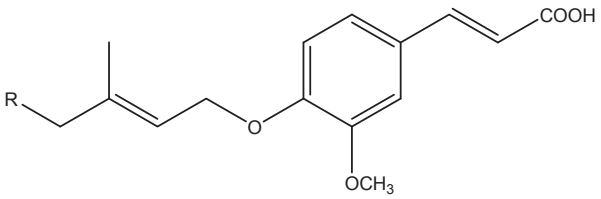

Figure 1. Structures of GOFA 1 (R = 3,3-dimethylallyl) and boropinic acid 2 (R = H)

GOFA has been isolated for the first time in 1966 from the bark extracts of *Acronychia baueri* Schott, an Australian small tree belonging to the family Rutaceae and has been shown to exert anti-inflammatory, neuroprotective, and dietary feeding colon cancer chemopreventive effects in rats and other properties closely related to cancer growth and development (Prager & Thregold 1966; Genovese & Epifano 2012; Shimizu *et al.* 2014). BPA 2 was isolated in 2000 by Ito from the aerial parts of the Australian plant *Boronia pinnata* Sm. (Rutaceae). This secondary metabolite showed valuable effects *in vitro* and *in vivo* as an inhibitor of the growth of *Helicobacter pylori* and as an anti-inflammatory agent (Curini *et al*. 200).

In continuation of our studies devoted to the analysis of citrus-based food preparations aiming to characterise the content of both GOFA and BPA, we wish to report herein a validated HPLC/UV-Vis method for the quantification of these ferulic acid derivatives in handmade citrus-based typical Italian liqueurs like limoncello, arancello, and mandarinetto.

MATERIAL AND METHODS

General experimental procedures. GOFA 1 and BPA 2 were chemically synthesised as already reported and their purity (> 98 %) was assessed by GC/MS and ^{1}H NMR (Epifano *et al*. 2011). Methanol (HPLC-grade) was purchased from Carlo Erba (Milan, Italy) and used without further purification. Double-distilled water was obtained by a Millipore Milli-Q Plus Waters treatment system (Millipore Bedford Corp., Bedford, USA). Solid phase extractions were carried out by Sep-Pak Vac 1cc (100 mg) C18 cartridges (Waters, Milford, USA). HPLC analyses were performed using a Waters liquid chromatograph equipped with a model 600 solvent pump and a 2996 photodiode array detector. Empower v. 2 software (Waters Spa, Milford, USA) was used for data acquisition. A C18 reversed-phase packing column (GraceSmart RP18, 4.6 × 150 mm, 5 µm; Grace, Deerfield, USA) was employed for the separation. The column was thermostatted at 10 ± 1°C using a Jetstream2 Plus column oven. The UV-Vis acquisition wavelength was set in the range of 210–600 nm. Analogue output channel A was set at a wavelength of 310 nm with a bandwidth of 9.6 nm. The qualitative analyses were performed at a wavelength of 288 nm. The injection volume was 20 µl. The mobile phase was directly on-line degassed using a Degassex DG-4400 apparatus (Phenomenex, Torrance, USA). The mobile phase composition consisted of double-distilled water (solvent A) and methanol (solvent B) at a flow rate of 1.2 ml/min following a gradient elution programme as reported in Table 1. The column re-equilibration was achieved in 7.39 min using the initial composition of the mobile phase employed for the two analytes separation.

Table 1. Gradient elution profile for HPLC analyses of citrus liqueurs

Time (min)	A (%)	B (%)
0.01	60	40
3.00	10	90
8.61	60	40
15	60	40

All the sample solutions were centrifuged and the supernatant was directly injected into the HPLC-UV/Vis system. Stock solutions of GOFA 1 and BPA 2 were independently prepared by dissolving the powders in MeOH at r.t. to provide an initial concentration of 1 mg/ml and stored in aliquots at –20°C in amber glass tubes. Working standard solutions were then obtained by appropriately diluting the stock solutions to obtain concentration values in the range of 0.5–50 µg/ml. These latter were also stored at –20°C in amber glass tubes for a period not longer than 4 weeks. The analyte-free extract was collected and pooled to get a sufficient volume to prepare external matrix-matched calibration standards and QC samples. Separate solutions were used to prepare calibration standards and QC samples.

Fruit collection and preparation of liqueurs. Limoncello was prepared with lemons collected in three different locations in Italy, namely Abruzzo, Emilia Romagna, and Molise regions. Arancello and mandarinetto were prepared with fruits collected in Abruzzo region. Voucher specimens named as L-ABR-001, L-ER-002, L-MOL-003, A-ABR-004, and M-ABR-005 have been stored in the deposit of

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the Laboratory of Phytochemistry and Chemistry of Natural Compounds at the Department of Pharmacy of the "G. D'Annunzio" University of Chieti-Pescara. All samples were identified by Dr. Francesco Epifano and Dr. Salvatore Genovese. Limoncello, arancello, and mandarinetto were then prepared following the procedure already described (Locatelli *et al*. 2012).

RESULTS AND DISCUSSION

Optimisation of LC separation. The best results in terms of peak response (area and shape), chromatographic resolution, and overall run time were obtained with a GraceSmart RP18 (4.6 mm × 150 mm, 5 μm) thermostatted at 10 ± 1°C. Under these experimental conditions, the recorded mean retention times were 7.5 ± 0.2 min and 6.2 ± 0.1 min for GOFA 1 and BPA 2, respectively. The HPLC profiles of the two external standards were found to be identical with those previously recorded for the same compounds (Genovese *et al*. 2014). Conditions set for the column purge and re-equilibration ensured stability for the column pressure and chromatogram background. The calculated capacity factors (*k'*) were 2.67 for BPA and 3.96 for GOFA. The dead retention time, calculated with uracil, was 1.83 minutes.

Very recently we have disclosed the presence of GOFA in grapefruit seeds employing a different analytical methodology in terms of the mobile phase composition (Taddeo *et al*. 2015). In this latter case the use of water/acetonitrile both acidified with 0.04% trifluoroacetic acid provided good results for the separation of this analyte. We have to modify the eluent composition turning to a water/methanol mixture in the absence of any acid as we observed the prompt formation of ethyl esters of both GOFA and BPA using the acidified aqueous solution cited above.

Limit of detection (LOD), limit of quantification (LOQ), and linearity. LODs were calculated by measuring S/N values obtained in the mobile phase spiked at a 0.2 μg/ml level and extrapolation of the corresponding values to S/N = 3. The calibration curves showed a good linearity in the concentration range of 0.5–50 μg/ml (r^2 = 0.9907 for GOFA 1 and r^2 = 0.9928 for BPA 2). The back-calculated calibration standard points showed R.S.D. percentage values ranging from –6.38% to –5.58%. The differences in percentage between the standard concentrations calculated from the calibration curves and the theoretical ones ranged from –12.2% to –2.1% for GOFA and from –10.8% to –3.7% for BPA. The limit of quantification (LOQ) was evaluated according to the guidance for industry on the validation of bioanalytical methods, as the lowest analyte concentration corresponding to a response at least 10 times higher than the blank response and that can be determined with 80–120% accuracy and 20% precision. The back-calculated concentration value, obtained from calibration curves, allowed to assess 0.5 μg/ml as the validated LOQ. This method is selective for the quantitative analyses because two wavelengths were used for the identification and quantification: 316 nm was used as the wavelength for the quantitative analyses and 288 nm for the qualitative ones. The structural assignment of both peaks was carried out by LC-MS. Recorded spectra were found to be identical to those previously reported for the same compounds (Genovese *et al*. 2010).

Precision and accuracy. The accuracy and precision results for both analytes have been obtained analysing QC samples prepared at three different concentration levels (1.0, 10, and 20 μg/ml). R.S.D. % values did not exceed 6% and 6.2% for GOFA and BPA, respectively. Bias values ranged from 0.24% to 10%.

Quantification of GOFA 1 and BPA 2. Results of the concentration of GOFA 1 and BPA 2 in limoncello, made with lemons of different geographical origin (Abruzzo, Emilia Romagna, and Molise), arancello, and mandarinetto are reported in the Table 2. A typical chromatogram of the analysis carried out on

Table 2. GOFA 1 and boropinic acid 2 concentrations in limoncello, arancello, and mandarinetto

Liqueur	GOFA (μg/ml ± SD)*	Boropinic acid (mg/g ± SD)*
Limoncello (Abruzzo)	1.68 ± 0.03	0.16 ± 0.01
Limoncello (Emilia Romagna)	0.30 ± 0.03	0.04 ± 0.01
Limoncello (Molise)	0.05 ± 0.01	nd
Arancello	0.11 ± 0.01	0.02 ± 0.01
Mandarinetto	0.08 ± 0.01	nd

*values expressed as μg/ml of liqueur; data are reported as mean ± SD (n = 10); nd – not detected

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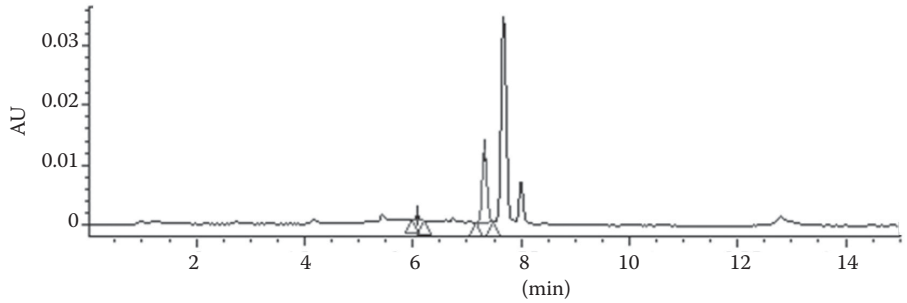

Figure 2. HPLC chromatogram of limoncello made with lemons collected in Abruzzo region

citrus liqueurs (e.g. limoncello from Abruzzo region) is shown in Figure 2.

Limoncello, arancello, and mandarinetto represent liqueurs deriving from a long-lasting maceration of citrus skins in high gradation ethanol. We have recently reported the chemical composition both in terms of GOFA and BPA of peel extract (deriving from a 24 h maceration in methanol preceded by ultrasonication of the finely triturated plant material in the same solvent) of nine *Citrus* spp. (Genovese *et al*. 2014). A comparison of the results obtained from the analysis of liqueurs with those obtained in our previous investigation clearly reveals substantial differences in qualitative and quantitative composition. GOFA and BPA deriving from a short lasting ultrasonication and maceration with methanol have been recorded in the order of mg/ml quantities while in both have been quantified liqueurs only in a µg/ml scale. Peels were seen to contain both analytes, in some instances in comparable quantities, while in some liqueurs (e.g. mandarinetto) BPA was not detected. These discrepancies may be due to the low efficiency of ethyl alcohol as the extracting solvent and/or to chemical degradation during the long period (up to 40 days) that is traditionally needed for the preparation of citrus liqueurs.

In this article we described an easy and effective extraction and HPLC procedures for the qualitative and quantitative analysis in three types of handmade typical Italian citrus-based liqueurs of two naturally occurring biologically active prenyloxycinnamic acids like GOFA 1 and BPA 2. To the best of our knowledge the findings described herein represent the first examples reported in the literature of the characterisation of the title phytochemicals in limoncello and similar spirit drinks. Limoncello, prepared using lemons from Abruzzo region, contains the highest concentrations of both GOFA (1.68 ± 0.03 µg/ml of liqueur) and BPA acid (0.16 ± 0.01 µg/ml). Only in two cases (e.g. limoncello made with lemons from Molise region and mandarinetto) BPA acid was not detected. A total recovery of both analytes strictly more than 100% was recorded for each liqueur under investigation. It is noteworthy to underline that in pure lemon, orange and mandarin juices, analysed under the same experimental conditions as above, GOFA and BPA acid having not been detected. This result may be explained by the recent discovery that the prenylation of phenylpropanoid cores is a biosynthetic enzymatic activity typically located in *Citrus* skins (Munakata *et al*. 2012). The occurrence of oxyprenylated secondary metabolites related to GOFA and BPA, namely farnesyloxy- and/or geranylgeranyloxy-derivatives, cannot be excluded, although there are no citations in the literature about the presence of such natural products in the genus *Citrus* (Epifano *et al*. 2013). In view of the well-documented pharmacological effects so far determined both *in vitro* and *in vivo* for GOFA and BPA, our analytical methodology may be also effectively used to evaluate their presence not only in citrus food preparations like juices, confectionery and beverages, but also and in other edible plants belonging to the families Rutaceae and Apiaceae (e.g. carrot, celery, fennel, parsnip, parsley, cumin, dill, chervil, and others). Such analyses are now in progress in our laboratories.

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