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Characterization and quantification of phenolic compounds of honeys from Sierra Nevada (Granada)

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Abstract: Antioxidant, anti-inflammatory and antimicrobial effects are some of the effects that have been attributed to honey, especially to its content of bioactive compounds, mainly phenolic compounds (PC), whose content varies greatly depending on the variety, origin, agronomic conditions, harvest season, and climate. The aim of the present study is to characterise 21 honeys from Sierra Nevada (Granada). High performance liquid chromatography coupled to quadrupole-time of flight mass spectrometry (HPLC-ESI-QTOF-MS) was used. The mass accuracy and true isotopic pattern in both MS and MS/MS spectra made possible the tentative identification of 58 PC including flavonoids, phenolic acids and derivatives. The average content of PC was 83.01±16.36 μg/g, with flavonoids accounting for more than 85%. The most abundant compounds were naringenin (16.88±3.15 μg/g), pinocembrin (12.33±2.92 μg/g), chrysin (12.21±2.09 μg/g), carnosol (9.52±2.90 μg/g), galangin (5.41±1.68 μg/g) and apigenin (5.24±0.89 μg/g). Due to their interesting composition, more studies are necessary to determine if the extreme environmental conditions from Sierra Nevada pose abiotic stress for the plants located there fostering the concentration in PC.

Keywords: honey; phenolic compounds; Sierra Nevada; HPLC-ESI-QTOF-MS

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1. Introduction

Honey, a natural product crafted by honey bees (Apis mellifera), is renowned for its nutritive and healthful qualities. Its composition exhibits considerable variability based on factors like botanical and geographical origin [1,2]. Honey primarily consists of sugars (constituting 80-85% of its composition), water (15-17%) and proteins (0.1-0.4%). Additionally, to a lesser extent, it contains enzymes, organic acids, vitamins, minerals and phenolic compounds, all of which significantly contribute to its sensory and functional attributes [3]. Honey has been associated with various beneficial effects, including antioxidant, anti-inflammatory and antimicrobial properties [4]. These health-promoting properties are particularly linked to its content of bioactive compounds, mainly phenolic compounds (PC). The PC content varies substantially depending on factors such as honey variety, origin, agronomic conditions, harvest season, and climate. Plants synthetize PC under both normal and stressful conditions, with functions that encompass attracting pollinating insects and safeguarding against pathogens and ultraviolet (UV) radiation [4]. Recent studies have reported a wide range of total phenolic content (TPC) values in honey, spanning from 6.5 ± 4.2 to 841.7 ± 304.0 μg/g [5]. While many researchers have analysed the content of bioactive compounds in some Spanish honeys, there are no

previous studies on the content of those compounds in honeys from Sierra Nevada (Granada), where the plants are exposed to extreme environmental conditions such as UV radiation, extreme temperatures, and altitude (hypoxia), which could influence the content of PC. Thus, the aim of the present study is to characterise and quantify the PC of 21 honeys from Sierra Nevada, which is a national park with a great variety of vegetation exposed to abiotic stresses that could increase the concentration and/or variety of PC contained in the honeys produced there.

2. Methodology

2.1. Extraction of phenolic compounds

The extraction of PC was performed using a previously described method [6,7] with some modifications. Sixty grams of each of the selected honeys were mixed with 150 ml of acidified water (pH 2.0). The mixture was stirred for 10 minutes at room temperature on a magnetic stirrer and then filtered through cotton wool to remove solid particles. The filtrate was mixed with 40 g Amberlite XAD-2 and stirred for 10 minutes at room temperature on a magnetic stirrer. The Amberlite particles were packed in a 33 cm long, 24 mm inner diameter gravimetric column (Sigma-Aldrich). Then, the column was washed with 100 ml of acidic water (pH 2.0) and then with 300 ml of milliQ water. In this way, PC present in the honey were retained in the column while sugars and other polar compounds were eluted with aqueous solvent. To collect the phenolic fraction, 300 ml of methanol were used. Then the phenolic fraction was dried in a rotary evaporator at a temperature of 30°C. The residue was dissolved in 5 ml of milliQ water and extracted in triplicate with diethyl ether (5 mlx3). The ether extracts were combined and the ether was removed at a rotary evaporator. The residue was dissolved in 1 ml of methanol, filtered through a 45µm membrane filter, transferred to an HPLC vial and frozen at -20°C until analysis.

2.2. Analysis of phenolic compounds

The analytical technique used was High Performance Liquid Chromatography coupled to Mass Spectrometry (HPLC-MS-MS/MS). Specifically, an Acquity HPLC system (Waters Corporation, Milford, MA, USA) coupled to an electrospray ionisation (ESI) source operating in negative mode and a quadrupole time-of-flight (QTOF) mass spectrometer (Waters) was used. To separate the compounds of interest effectively, it was used an ACQUITY UPLC BEH Shield RP18 column (1.7 µm, 2.1 x 100 mm; Waters Corporation, Milford, MA, USA). Acidified H_2O with 1% acetic acid and methanol as solvents A and B, respectively, were used for the mobile phases. A linear gradient was applied so that at the start 95% was solvent A and 5% solvent B; at minute 30, 23.9% was solvent A and 76.1% solvent B; and at minute 33, 100% was solvent B. The initial conditions were maintained for 6 minutes before each analysis. The column temperature was maintained at 40°C and the injection volume was 0.5ml/min.

2.3. Characterisation of phenolic compounds

MassLynx 4.1 software (Waters Corporation, Milford, MA, USA) was used to process the chromatographic data. The characterisation strategy was based on the exact mass and fragment information provided by the compound-specific MS and MS/MS spectra determined by the QTOF mass analyser. For the acquisition of information on the chemical structure of the compounds, in addition to consulting previously published research, the following databases were used: SciFinder Scholar (http://scifinder.cas.org), FoodDb (https://foodb.ca/), MassBank (http://massbank.jp), Pubchem (https://pubchem.ncbi.nlm.nih.gov), Metfrag (https://msbi.ipb-halle.de/MetFrag/), METLIN (http://metlin.scripps.edu), National Institute of Standards and Technology (https://www.nist.gov/), and the National Institute of Health (NIH) database. Additionally, SIRIUS 4 (https://bio.informatik.uni-jena.de/sirius/) was used to obtain metabolite

structure information. Six analytical standards (catechin, chlorogenic acid, ferulic acid, rutin, phloridizin, quercetin, phloretin and vanillic acid) were employed to estimate the amount of phenolic compounds present in the honeys.

3. Results and discussion

Fifty-eight phenolic compounds were characterized in the 21 honeys, including flavonoids, phenolic acids and derivatives (Table 1).

Table 1. Phenolic compounds characterised in the 21 honeys.

$[M-H]^-$	RT	Molecular formula	Proposed compound	Fragments	References
135.0433	2.189	C8H8O2	Phenylacetic acid	117	[18]
135.0438	2.01	C8H8O2	Vinylcatechol	134, 133, 105	[19]
137.0222	4.014	C7H6O3	Hydroxybenzoic acid	93	[20]
153.0208	4.852	C7H6O4	Protocatechuic Acid	109, 137	[21]
163.0386	3.225	C9H8O3	Cumaric acid (Isomer 1)	145, 119	[20]
163.0396	3.341	C9H8O3	Cumaric acid (Isomer 2)	119, 117	[20]
165.0547	2.458	C9H10O3	4-hydroxicinnamic acid	161, 133, 132, 122	[22]
165.0558	3.448	C9H10O3	L-(-)-phenylactic acid	147	[23]
167.0337	2.065	C8H8O4	Homogentisic acid	134, 137, 131, 117, 108	[24]
177.0181	1.769	C9H6O4	Esculatin	145, 125, 120, 144	[25]
193.0495	2.095	C10H10O4	Coniferic/ferulic acid	133	[26]
195.0659	1.995	C10H12O4	4-methoxyphenylactic acid	133, 177, 149	[27]
197.0442	2.16	C9H10O5	Siringic acid (Isomer 1)	106	[20]
197.0455	2.262	C9H10O5	Siringic acid (Isomer 2)	121, 123	[20]
211.0599	4.875	C10H12O5	Methylsyringate	181	[28]
221.0804	3.45	C12H14O4	3-hydroxy-1-(2-methoxyphenyl)penta-1,4-dione (Isomer 1)	133	[29]
221.0811	3.605	C12H14O4	3-hydroxy-1-(2-methoxyphenyl)penta-1,4-dione (Isomer 2)	133	[29]
223.0609	3.228	C11H12O5	Sinapic acid	144, 116, 142, 160	[21]
223.0968	6.851	C12H16O4	Vanillin 1,2-butylene glycol	151, 136, 108	[30]
253.0497	12.928	C15H10O4	Chrysin	209, 143, 145, 119,195	[31]
255.0659	12.128	C15H12O4	Pinocembrin	171, 133, 213, 134, 169	[32]
269.0439	10.498	C15H10O5	Apigenin	117,149, 201, 145, 183, 107	[33]

269.0441	9.803	C15H10O5	Baicalein	129, 143, 151	[25]
269.0445	13.681	C15H10O5	Galangin	211, 239, 195,167, 151	[34]
271.0600	7.979	C15H12O5	Pinobanksin	253, 197,225, 209, 125	[35]
271.0603	8.406	C15H12O5	Naringenin	253,197, 161,125, 225	[20]
283.0597	11.218	C16H12O5	Prunetin	211, 238, 167, 165	[36]
283.0604	13.878	C16H12O5	Biochanin A	268, 211, 239, 269, 195	[37]
283.0605	13.469	C16H12O5	Genkwanin	134, 175, 168, 148, 159	[38]
283.0969	13.161	C17H16O4	Phenylethyl caffeate	135, 133, 161, 134	[34]
285.0381	10.787	C15H10O6	Kaempferol (Isomer 1)	151, 184, 245, 255, 273	[39]
285.0392	6.831	C15H10O6	Luteolin (Isomer 1)	151, 257	[40]
285.0394	13.682	C15H10O6	Kaempferol (Isomer 2)	269, 268, 211, 239	[39]
285.0396	8.691	C15H10O6	Luteolin (Isomer 2)	255, 133, 283, 151	[40]
285.0404	7.646	C15H10O6	Kaempferol (Isomer 3)	255, 227, 211, 284	[39]
285.0408	9.135	C15H10O6	Luteolin (Isomer 3)	241, 133	[40]
285.0772	12.291	C16H14O5	5-O-Methylnaringenin	188, 191, 255, 243, 158	[20]
287.0555	5.52	C15H12O6	Eriodictyol	161, 269, 251	[41]
299.0545	11.479	C16H1206	Kaempferide (Isomer 1)	284, 227, 256, 165, 269	[42]
299.0547	11.155	C16H1206	Kaempferide (Isomer 2)	284	[42]
301.0322	4.236	C15H10O7	Quercetin	175, 183, 201, 225, 245	[42]
301.0339	4.077	C15H10O7	Quercetin	255, 273, 213, 151	[42]
301.0354	9.343	C15H10O7	Morin	273, 151, 257, 178, 255	[34]
301.0696	9.9400	C16H14O6	Hesperetin (Isomer 1)	164	[43]
301.0713	5.3190	C16H14O6	Hesperetin (Isomer 2)	151, 177	[43]
301.0717	5.4440	C16H14O6	Hesperetin (Isomer 3)	177, 286	[43]
301.2008	8.5460	C15H10O7	Tricetin	255, 151	[44]
315.0487	10.875	C16H12O7	3-Methylquercetin/Isorhamnetin (Isomer 1)	241, 242, 270, 313, 300	[39]
315.0500	10.157	C16H12O7	3-Methylquercetin/Isorhamnetin (Isomer 2)	300	[39]
315.0506	9.136	C16H12O7	3-Methylquercetin/Isorhamnetin (Isomer 3)	241, 242, 270, 271, 300, 313	[39]

329.0650	9.824	C17H14O7	Quercetin dimethyl ether (Isomer 1)	314	[45]
329.0664	11.956	C17H14O7	Quercetin dimethyl ether (Isomer 2)	314	[45]
329.1744	15.415	C20H26O4	Carnosol (Isomer 1)	285	[35]
329.1745	15.068	C20H26O4	Carnosol (Isomer 2)	285	[35]
329.1753	15.708	C20H26O4	Carnosol (Isomer 3)	285	[35]
329.1758	14.856	C20H26O4	Carnosol (Isomer 4)	285	[35]
431.0974	7.651	C21H20O10	Kaempferol-rharmnoside	285, 255, 227	[46]
461.1065	9.049	C22H22O11	8-Methoxykaempferol 7-rhamnopyranoside	287, 299, 315, 259, 139	[46]

RT: retention time.

The average TPC from the Sierra Nevada honeys analysed was 83.01±16.36 μg/g, being above the average of other Spanish honeys such as Galician honeys, which contain an average of 38 μg/g [8]. Flavonoids accounted for more than 85% of the TPC. Figure 1 shows the content of phenolic acids, flavonoids, other phenolic compounds and total phenolic compounds of each honey. The most abundant compounds were naringenin (16.88±3.15 μg/g), pinocembrin (12.33±2.92 μg/g), chrysin (12.21±2.09 μg/g), carnosol (9.52±2.90 μg/g), galangin (5.41±1.68 μg/g) and apigenin (5.24±0.89 μg/g). Naringenin is noted for its positive effects on the cardiovascular system through antioxidant, anti-inflammatory, anti-atherogenic and anti-apoptotic actions [9]. Pinocembrin is a flavanone with antioxidant, antimicrobial and anti-inflammatory properties, and has recently been studied for its potential to inhibit histidine decarboxylase as a new natural anti-allergic drug candidate [10]. Chrysin has shown significantly greater antiproliferative activity on cancer cell growth than other compounds [11]. It also has antioxidant, anti-obesity, anti-inflammatory, anti-diabetic and neuroprotective activity [12]. Carnosol is a phenolic diterpene with demonstrated antioxidant, anti-inflammatory and anticancer activity [13,14]. Positive effects of carnosol have also been reported in ischaemic stroke, by inhibiting apoptosis and attenuating oxidative damage and cellular inflammation [15]. The main positive effects of galangin are attributed to its anti-inflammatory, antioxidant, anticancer and antineoplastic properties [16]. Apigenin has shown therapeutic functions through cell cycle arrest, apoptosis, anti-inflammatory. In addition, apigenin contributes to counteracting oxidative stress by enhancing the expression of anti-oxidant enzymes such as glutathione synthase, catalase and superoxide dismutase. After its absorption into the digestive tract, apigenin is able to reach the brain and could have antidepressant and antianxiety effects [17].

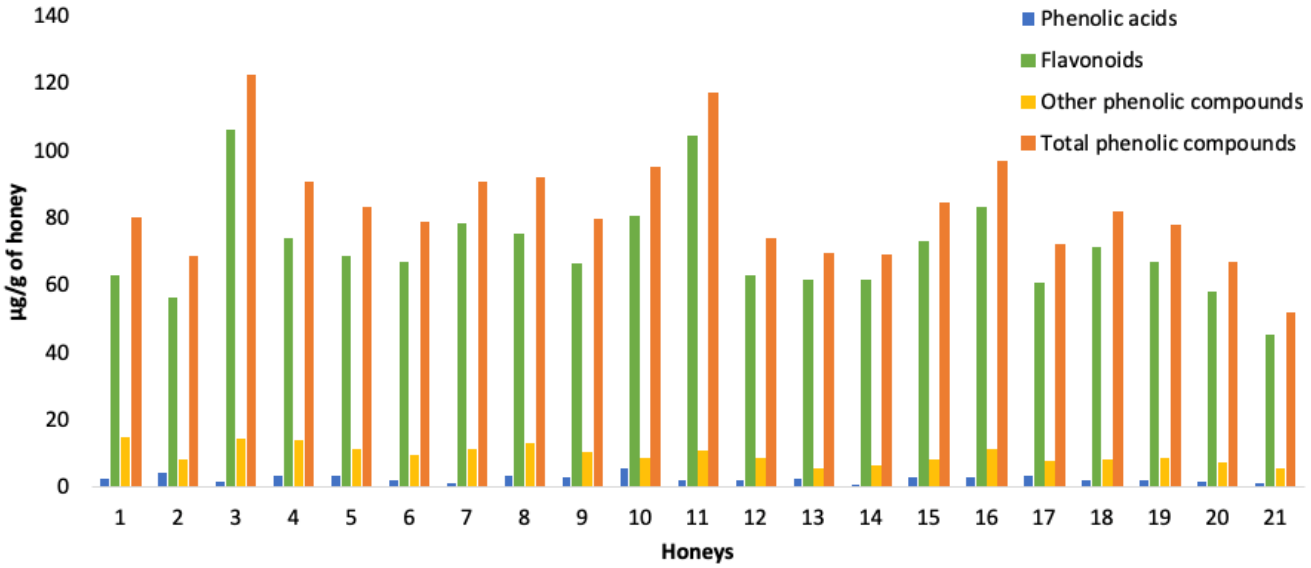

Figure 1. Content of phenolic compounds of Sierra Nevada honeys.

4. Conclusion

Due to their interesting composition, more studies are necessary to determine if the peculiar environmental conditions from Sierra Nevada, such as UV radiation, extreme temperature, or altitude (hypoxia) pose abiotic stress for the plants located there fostering the concentration in PC and thus increasing the antioxidant, antimicrobial, anti-inflammatory and anticarcinogenic activity of these honeys.

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