Flavonol Glycosides from Distilled Petals of *Rosa damascena* Mill.

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Flavonol glycosides were extracted from petals of *Rosa damascena* Mill. after industrial distillation for essential oil recovery and characterized by high-performance liquid chromatography-electrospray ionization mass spectrometry. Among the 22 major compounds analyzed, only kaempferol and quercetin glycosides were detected. To the best of our knowledge, the presence of quercetin 3-*O*-galactoside and quercetin 3-*O*-xyloside has so far not been reported within the genus *Rosa*. In addition, based on their fragmentation patterns, several acylated quercetin and kaempferol glycosides, some of them being disaccharides, were identified for the first time. The kaempferol glycosides, along with the kaempferol aglycone, accounted for 80% of the total compounds that were quantified, with kaempferol 3-*O*-glucoside being the predominant component. The high flavonol content of approximately 16 g/kg on a dry weight basis revealed that distilled rose petals represent a promising source of phenolic compounds which might be used as functional food ingredients, as natural antioxidants or as color enhancers.

Key words: Rosa damascena, Flavonols, HPLC-MS

Introduction

Rose essential oil, also known as rose otto, is a highly prized product used in perfumery, cosmetics and pharmacy (Kaul *et al.*, 2000; Kovats, 1987; Umezu *et al.*, 2002). Bulgaria and Turkey are the main rose processing countries in the world which extract the rose oil by water-steam distillation of *Rosa damascena* Mill. petals. Since more than 3,000 kg of petals yield 1 kg of rose oil and 1 kg of the fresh raw material gives approximately 2 kg of residue on a wet weight basis, several thousand tons of waste material annually result from the distilleries in Bulgaria alone. Due to the selective rose oil recovery, without using solvent extraction, the polar phenolic compounds are retained in the waste material. The evaluation of its potential as a source of polyphenolic extracts with specific health-beneficial effects for development of functional foods requires an accurate and reliable chemical characterization of individual compounds, which is a prerequisite to assess their contribution to the total antioxidant activity as determined by different *in vitro* tests (Becker *et al.*, 2004). Up to now, the phenolic compounds of rose petals, particularly the flavonoids, have been studied with respect to their anthocyanin copigmentation, explaining the stability of rose flower color (Asen *et al.*, 1971; Biolley and Jay, 1993). However, the polyphenolic profile has not yet precisely been assigned because of the limited availability of standard compounds and the similar spectral characteristics of structurally related components. Moreover, quantification was often restricted to the aglycones after acid hydrolysis of the extracts (Helsper *et al.*, 2003) or to the total flavonoid content, as determined by differential photometric measurement of the extracts (Gonnet, 2003). Therefore, the aim of the present study was to determine the amounts of individual phenolic compounds from distilled rose petals employing high-performance liquid chromatography with diode array and mass spectrometric detection. Since our preliminary experiments have shown that the polyphenolic profile was almost completely composed of flavonol glycosides, particular attention was given to their separation and characterization.

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Materials and Methods

Plant material

Wet rose petals (~ 10% dry matter) originating from steam distillation of *Rosa damascena* Mill. were supplied by Bulgarska Roza AD (Karlovo, Bulgaria). The waste material (~ 5 kg) was pressed in a rack and cloth press and the pomace obtained was hot air-dried in a cross-flow dryer (60 °C, 6 h).

Chemicals

Standards used for HPLC and MS identification were: quercetin 3-*O*-xyloside (Plantech, Reading, UK); quercetin and kaempferol (Roth, Karlsruhe, Germany); quercetin 3-*O*-galactoside, quercetin 3-*O*-glucoside, quercetin 3-*O*-rhamnoside, quercetin 3-*O*-rutinoside and kaempferol 3-*O*-glucoside (Extrasynthese, Lyon, France). All solvents were purchased from VWR International GmbH (Darmstadt, Germany) and were of analytical or gradient grade.

Sample preparation

According to Schieber *et al.* (2003a), aliquots of 2.0 g of the finely ground rose pomace were mixed with 0.5 g of ascorbic acid in an amber glass round-bottomed flask and extracted with 50 ml of aqueous acetone (80%, v/v) in a nitrogen atmosphere for 3 h under stirring at ambient temperature. The extraction mixture was centrifuged (10 min, 3,480 × *g*), and the residue was extracted with 50 ml of aqueous acetone for 10 min. The organic solvent was removed from the combined supernatants by evaporation *in vacuo* at 30 °C. The residual aqueous solution was transferred into a graduated flask and made up to 100 ml with deionized water. After filtration through a fluted filter, 6 ml of filtrate were used for further purification. Polyamide CC6 (2 g, 0.05–0.16 mm, Macherey-Nagel, Dueren, Germany) was filled into an Econo-Pac column (BioRad, Munich, Germany) and successively conditioned with 25 ml of methanol and 50 ml of deionized water prior to application of the extract to the column. After washing with water (50 ml), the polyphenolic fraction was recovered by elution with methanol (50 ml). The eluate was evaporated to dryness, and the residue was dissolved in 2 ml of methanol. The solution was membrane-filtered (0.45 μm, Whatman Inc., Clifton, USA) and used for HPLC.

HPLC analysis

The separation of phenolic compounds was performed using an Agilent (Waldbronn, Germany) HPLC series 1100 system equipped with ChemStation software, a model G1322A degasser, a model G1312A binary gradient pump, a model G1329/1330A thermoautosampler, a model G1316A column oven, and a model G1315B diode array detector. The column used was a 150 × 3.0 mm i.d., 4 μm Synergi Hydro-RP (Phenomenex, Torrance, USA) with a 4.0 × 2.0 mm i.d. C18 ODS guard column, operated at 25 °C. The mobile phase consisted of 2% (v/v) acetic acid in water (eluent A) and of 0.5% acetic acid in water and acetonitrile (50:50, v/v; eluent B). The gradient program was as follows: 10% B to 55% B (50 min), 55% B to 100% B (10 min), 100% B to 10% B (5 min). The injection volume for all samples was 4 μl. Monitoring was performed at 370 nm at a flow rate of 0.4 ml/min. Spectra were recorded from 200 nm to 600 nm (peak width 0.2 min).

LC-MS analysis

LC-MS analysis was performed with the HPLC system described above connected in series with a Bruker (Bremen, Germany) model Esquire 3000+ ion trap mass spectrometer fitted with an ESI source. Negative ion mass spectra of the column eluate were recorded in the range *m/z* 50–2800. Nitrogen was used as the dry gas at a flow rate of 9.0 l/min and at a pressure of 40.0 psi. The nebulizer temperature was set at 365 °C. Collision-induced dissociation (CID) spectra were obtained with a fragmentation amplitude of 1.0 V (MS/MS). Helium was used as the collision gas (1.2×10^{-5} mbar).

Quantification of individual compounds

Individual flavonols were quantified using a calibration curve of the corresponding standard compound. When reference substances were not available, the calibration of structurally related compounds was used, including a molecular weight correction factor (Chandra *et al.*, 2001). Sample preparation and HPLC determination were performed in duplicate. Data are expressed as mean ± standard deviation.

Results and Discussion

Identification of flavonol glycosides by LC-MS

A C18 stationary phase with hydrophilic endcapping was used. It has been demonstrated to be highly suitable for an efficient separation of polyphenols from various plant matrices applying MS compatible liquid chromatography systems (Hilt *et al.*, 2003; Kammerer *et al.*, 2004; Schieber *et al.*, 2001; Schütz *et al.*, 2004). Among the 22 major compounds analyzed, only kaempferol and quercetin glycosides were detected, which is consistent with the results of other studies on the flavonol profile of rose petals (Asen *et al.*, 1971; Nayeshiro and Eugster, 1989). However, due to the similar UV spectrum of individual components belonging to the same polyphenolic subclass and because of the limited availability of reference compounds, especially kaempferol glycosides, HPLC coupled to mass spectrometry proved to be extremely helpful for reliable peak identification in the present study.

Compounds **2**–**5**, **19** and **22** were unambiguously identified as quercetin glycosides and the quercetin and kaempferol aglycones, respectively, by comparison of their retention times and UV spectral characteristics with those of standard substances. The mass-to-charge ratios of the pseudomolecular ions ($[M\text{-}H]^-$) and the fragments released after CID in the MS^2 experiment confirmed the peak assignment (Table I). To the best of our knowledge, the presence of quercetin 3-*O*-galactoside (**3**) and quercetin 3-*O*-xyloside (**5**) has so far not been reported within the genus *Rosa*, which may be of chemotaxonomic interest. Compound **9** exhibited both quercetin 3-*O*-rhamnoside and kaempferol 3-*O*-glucoside fragmentation patterns. In contrast, its UV spectrum fully matched the kaempferol 3-*O*-glucoside characteristics, which is indicative of the low abundance of quercetin 3-*O*-rhamnoside. In further experiments, by separation of these two compounds, achieved under modified chromatographic conditions (Schieber *et al.*, 2003a; Schütz *et al.*, 2004), the presence of only trace amounts of quercetin 3-*O*-rhamnoside was confirmed, while kaempferol 3-*O*-glucoside was the most abundant component. However, since the resolution of most of the other peaks was adversely affected, subsequent quantification of kaempferol 3-*O*-glucoside was performed by integration of the area of peak 9 without corrections.

Table I. Characteristic data and contents of flavonol glycosides from distilled rose petals.

Compound	Identity	Retention time [min]	HPLC-DAD λ_{max} [nm]	$[M\text{-}H]^-$ *m/z*	MS^2 fragments *m/z*	Content [mg/kg dw]
1	Quercetin galloylhexoside	30.0	231, 256, 263sh, 302sh, 353	615	463/301	60 ± 5
2	Quercetin 3-*O*-rutinoside	32.6	231, 256, 264sh, 302sh, 354	609	301	117 ± 29
3	Quercetin 3-*O*-galactoside	33.1	231, 256, 264sh, 302sh, 353	463	301	1228 ± 32
4	Quercetin 3-*O*-glucoside	33.9	231, 256, 263sh, 302sh, 353	463	301	883 ± 73
5	Quercetin 3-*O*-xyloside	35.8	231, 256, 264sh, 302sh, 354	433	301	137 ± 1
6	Kaempferol hexoside	37.0	231, 265, 300sh, 347	447	284	1331 ± 43
7	Kaempferol disaccharide	37.4	232, 265, 300sh, 348	593	285	587 ± 26
8	Quercetin disaccharide	37.9	231, 256, 264sh, 302sh, 354	609	301	476 ± 18
9a	Quercetin 3-*O*-rhamnoside	38.9	–	447	301	Trace
9b	Kaempferol 3-*O*-glucoside	38.9	231, 265, 300sh, 347	447	285	4156 ± 61
10	Kaempferol hexoside	40.0	231, 265, 298sh, 347	447	284	74 ± 2
11	Kaempferol galloylhexoside	41.1	231, 265, 300sh, 347	599	285	301 ± 9
12	Kaempferol pentoside	41.7	232, 265, 300sh, 347	417	284	521 ± 53
13	Kaempferol disaccharide	43.2	231, 265, 301sh, 348	593	285	439 ± 23
14	Kaempferol pentoside	43.5	232, 265, 298sh, 348	417	285	828 ± 40
15	Kaempferol deoxyhexoside	44.9	231, 265, 300sh, 347	431	285	743 ± 33
16	Quercetin acetyldisaccharide	46.4	230, 258, 264sh, 298sh, 354	651	609/301	88 ± 1
17	Quercetin disaccharide	49.7	231, 256, 264sh, 302sh, 354	609	301	58 ± 1
18	Kaempferol acetyldisaccharide	51.9	231, 265, 300sh, 348	635	593/285	492 ± 11
19	Quercetin	52.9	230, 255, 266sh, 302sh, 371	301	179/151	189 ± 50
20	Kaempferol disaccharide	55.1	231, 265, 300sh, 347	593	285	1723 ± 60
21	Kaempferol disaccharide	56.2	231, 265, 301sh, 348	593	285	204 ± 6
22	Kaempferol	59.9	231, 265, 301sh, 367	285	257	1339 ± 39

Based on their UV spectrum and fragmentation behavior, four compounds (**6**, **10**, **12**, **14**) were tentatively identified as kaempferol glycosides. While components **6** and **10** exhibited a fragmentation pattern very similar to that of kaempferol 3-*O*-glucoside and were thus assigned to kaempferol hexosides, the mass spectrometric characterization of compounds **12** and **14** provided evidence for the presence of kaempferol pentosides. Consistent with the CID patterns of flavonoid glycosides (Hvattum and Ekeberg, 2003), a homolytic cleavage was observed, generating a radical aglycone ion (*m/z* 284) as the main fragment from compounds **6**, **10**, and **12**. All other kaempferol glycosides detected in the present study produced a main fragment at *m/z* 285, indicating a heterolytic cleavage of the sugar moiety. Similar fragmentation behavior has recently been reported for flavonol glycosides from mango peels (Schieber *et al.*, 2003a). Another kaempferol glycoside, **15**, showed a loss of 146 Da in the MS^2 experiment, which could be attributed to a deoxyhexose moiety. In contrast to a recent study on the polyphenolic composition of *Euterpe oleracea* Mart. fruit (Gallori *et al.*, 2004), where taxifolin deoxyhexoside has been described as *C*-linked, due to the absence of the fragment corresponding to the aglycone, the characteristic aglycone product ion at *m/z* 285 was observed in our MS^2 experiment, which suggests the presence of an *O*-linked kaempferol deoxyhexoside. Furthermore, two compounds (**8**, **17**), displaying pseudomolecular ions at *m/z* 609, showed fragmentation data and UV spectral characteristics identical to those of quercetin 3-*O*-rutinoside (rutin). It is therefore concluded that these quercetin glycosides are also composed of a disaccharide moiety consisting of a hexose and a deoxyhexose. Accordingly, four compounds (**7**, **13**, **20**, **21**) yielding pseudomolecular ions at *m/z* 593 and prominent fragments at *m/z* 285 were identified as kaempferol disaccharides. Analogously, flavonol disaccharides different from rutin have recently been described as natural substances in stem bark extracts of erect spiderling (*Boerhavia erecta* L.) and spiny amaranth (*Amaranthus spinosus* L.) (Stintzing *et al.*, 2004). However, flavonol disaccharides other than quercetin and kaempferol 3-*O*-rutinoside have not yet been reported in rose petal extracts. In addition, several acylated flavonol glycosides were tentatively identified for the first time. CID of compound **1** produced a main fragment at *m/z* 463, corresponding to a loss of 152 Da, which is indicative of a galloyl moiety. Furthermore, according to the second prominent fragment at *m/z* 301, a loss of 314 Da was observed, which probably results from a moiety composed of a hexose and a gallic acid. Thus, compound **1** was identified as quercetin galloylhexoside. This peak assignment corresponds to the highly hydrophilic nature of compound **1**, explaining the low retention of this substance. Accordingly, compound **11**, exibiting a loss of 314 Da in the MS^2 experiment, was identified as kaempferol galloylhexoside. Similar galloylated flavonol glycosides isolated from the leaves of *Pemphis acidula* Forst. (Lythraceae) have been demonstrated to be more potent antioxidants than the corresponding non-galloylated glycosides (Masuda *et al.*, 2001). CID of compounds **16** and **18** led to the loss of 42 Da indicative of an acetyl moiety. Whereas component **16** produced a main fragment at *m/z* 609, compound **18** yielded a prominent fragment at *m/z* 593. Moreover, the formation of characteristic aglycone product ions at *m/z* 301 and at *m/z* 593, respectively, confirmed that the acetyl group is linked to the sugar unit. Therefore, these components were identified as quercetin (**16**) and kaempferol (**18**) acetyldisaccharides. Although acetylated flavonol glycosides have also been isolated from Ranunculaceae (Fico *et al.*, 2000) and Fabaceae (Foo *et al.*, 2000), their occurrence in Rosaceae has not yet been described.

Quantification of individual compounds

As can be seen from Table I, the kaempferol glycosides, along with the kaempferol aglycone, accounted for 80% of the compounds that were quantified, with kaempferol 3-*O*-glucoside (**9b**) being by far the predominant component. Among the quercetin glycosides quercetin 3-*O*-galactoside (**3**) was the major compound. Comparison of these results with those obtained in other studies dealing with the flavonol content of rose petals is difficult, due to differences in the analytical methods and between the rose species. Futhermore, the industrial rose oil distillation process may also affect the stability of flavonol glycosides. The latter assumption may explain the relatively high quantities of the aglycones, especially of kaempferol, observed in the present study. Due to the short rose oil extraction campaign resulting in large amounts of wet waste material, conventional drying may be required for their preservation. As has recently

been demonstrated with apple pomace (Schieber *et al.*, 2003b), flavonol glycosides remain virtually unaffected by the industrial drying process. Therefore, significant changes of flavonols are hardly to be expected during drying of distilled rose petals.

The total polyphenolic content of the remaining distillation material was approximately 16 g/kg on a dry weight basis, which exceeded that of other residual products such as apple (Schieber *et al.*, 2003b) and artichoke (Schütz *et al.*, 2004) pomace. Moreover, it should be noted that the polyphenolic composition was almost completely dominated by flavonols, which have been demonstrated to be one of the most potent polyphenolic antioxidants (Foti *et al.*, 1996). Therefore, distilled rose petals represent a rich source of phenolic compounds that might be used as health-promoting ingredients of functional foods and dietary supplements or as natural antioxidants, an alternative to the synthetic antioxidants used in food industry and cosmetics. From a technological point of view, the enhancement and stabilization of the color of fruit juices (Rein and Heinonen, 2004; Talcott *et al.*, 2003) and anthocyanin-based coloring foodstuffs (Malien-Aubert *et al.*, 2001) by copigmentation with extracts from rose petals should also be considered, especially since flavonols have been shown to be highly effective even at a low copigment/pigment ratio (Asen *et al.*, 1972). Additionally, various pharmaceutical applications may also be taken into consideration (Cho *et al.*, 2003; VanderJagt *et al.*, 2002).

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