Flavonoids from the leaves of *Ribes nigrum* L. identification of a malonyl flavonol and HPLC analysis

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Introduction

The *Ribes nigrum* leaves are traditionally used for its diuretic and anti-inflammatory activities (Racz-Kotilla *et al.*, 1977; Chanh *et al.*, 1986; Declume, 1986; Pommier, 1990; Serrano *et al.*, 1990; Mongold *et al.*, 1993).

Flavonoids are the main constituents of the leaf : quercetin, kaempferol, myricetin and isorhamnetin glycosides have been identified (Anton, 1999; Calamita *et al.*, 1983; Gaspodinava *et al.*, 1966).
The flavonoids are used in the control of this drug: identification and dosage.
Till now, the presence of acylated flavonoids has never been detected in this plant.
In this work, we report for the first time, the isolation of kaempferol-3-O-(6″-O-malonyl)-ß-D-glucopyranoside (KGM), in the leaves of black-currrant.
An HPLC method for the qualitative and quantitative determination of isoquercitrin, astragalin and KGM in ribes leaves is described.

Material and methods

Plant material

Ribes nigrum leaves were provided by the laboratory "La Tisane Provençale" (Aubagne, France).

Isolation and structure determination of KGM

Powdered dried leaves (10 g) were extracted with 500 ml of 60 % aqueous methanol.
The residual aqueous layer was successively extracted with methylethylketone.
The methylethylketone extract (1.5 g) was subjected to polyamide column chromatography (50 g, polycarpolactam, 0.07 mm, Macherey-Nagel) and eluted with methanol (250 ml), ethanol (250 ml), acetone-water (8:2,v/v, 2.5 l).
The acetone-water extract furnished 49 mg of pure KGM.
The ^{1}H and ^{13}C-NMR spectra were recorded on a Bruker AMX400 spectrometer in CD_3OD solution, internal reference was TMS. FAB-Mass spectra were obtained from Nermag R-10-10H mass spectrometer in the negative ion mode.

HPLC analysis

Apparatus: Waters HPLC system (Milford, MA, USA):
- Software: Millenium^{32}, v. 3.01
- Two solvent delivery systems 510
- Photodiode array detector 996
- Autosampler WISP 717

Analytical conditions
- Analytical column Novapak C-18 (Waters), 4 µm, 150 x 3.9 mm + Guard Pak Novapak insert
- Mobile phase:
  solvent A: H_2O/H_3PO_4 (100:0.3, v/v)
  solvent B : $CH_3CN/H_2O/H_3PO_4$ (80:20:0.3, v/v)
  The solvent gradient profile was: 0-30 min: 17-25 % B; 30-35 min: 25-30 % B; 35-45 min: 30 % B.
- flow rate: 0.7 ml-min^{-1}
- UV detection: 340 nm
- Run time: 46 min
- Injection volume: 20 µl of filtered standards and samples.

Samples
- Standard solutions: 1.0-8.0 mg of astragalin, isoquercetrin and KGM were dissolved in 100 ml 60 % aqueous methanol.
- Extract solutions: 0.45 g-3.60 g of powdered leaves were

extracted under reflux with 90 ml of 60 % aqueous methanol for 30 min. The solutions were filtered and completed to 100 ml with the same solvent.

Results

Identification of KGM

From the methylethylketone extract, the kaempferol-3-O-(6"-O-malonyl)-ß-D-glucopyranoside was isolated by column chromatography on polyamide. This malonated flavonol glycoside was identified by comparison of its ^{13}C and ^{1}H-NMR spectra with published data (Wald *et al.*, 1989; Veit *et al.*, 1990).
The FAB-Mass spectra of this compound showed a molecular ion peak at m/z 533 [MH]- corresponding to a molecular formula of $C_{24}H_{22}O_{14}$.
This flavonoid was obtained for the first time from *Ribes nigrum* L. (figures 1 and 2)

Chromatography

Under the chromatographic conditions described, very satisfactory results were obtained within 46 min for the identification of rutin, isoquercitrin, astragalin and KGM (figure 1).

Validation

The validation data are presented in Table 1.
The linearity of the calibration curve was achieved between 0.01-0.08 mg-ml^{-1} for standards and between 0.45-3.0 g-100 ml^{-1} for black currant leaves.
Calibration curve were plotted by correlating peak areas against the corresponding concentrations. The assay was linear in the concentration range studied ($r > 0.999$).

The precision of the method was tested by both intra-day (n=6) and inter-day (3 days, n=6) reproducibilities from the aqueous methanolic extract of Ribes nigrum leaves, at a concentration of 1.80.100 ml^{-1} (Table 1).

The CV values were less than 2.25 % for intra-day assays and 1.92 % for inter-day assays.
The accuracy values showed very satisfactory results for precision: % of recovery ≈ 101 % (CV<2%).

Dosage

Three samples of *Ribes nigrum* leaves from different suppliers have been tested.
Chromatic profiles and content of isoquercitrin, astragalin and KGM were determined and compared (Table 2). The chromatographic profiles obtained from the samples were similar.
The comparison of the concentration of the quantified flavonoids showed that the content in kaempferol-3-glucomalonyl was eight times higher than the content in isoquercitrin and astragalin.

Conclusion

The *Ribes nigrum* leaves are currently used in phytotherapy for its anti-inflammatory properties. Flavonoids are chosen for the drug identification and dosage.

Phytochemical interest: we report, for the first time, the isolation and the identification of a malonated flavonol glycoside : kaempferol-3-O-(6"-O-malonyl)-β-D-glucopyranoside in the *Ribes nigrum* leaves.
Analytical interest: for the first time an HPLC method for the dosage of these flavonoids in black currant is reported. This method allowed a complete separation and a simultaneous determination of isoquercitrin, astragalin and KGM. This highly selective HPLC method can be proposed for the qualitative and quantitative control of the drug, plant extracts or phytomedicines prepared from Ribes nigrum leaves.

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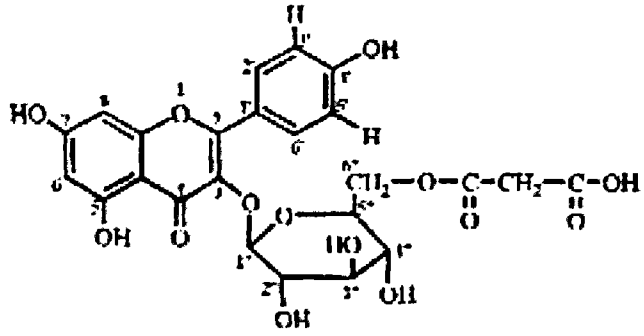

Figure 1. Kaempferol-3-O-(6″-O-malonyl)-ß-D-glucopyranoside

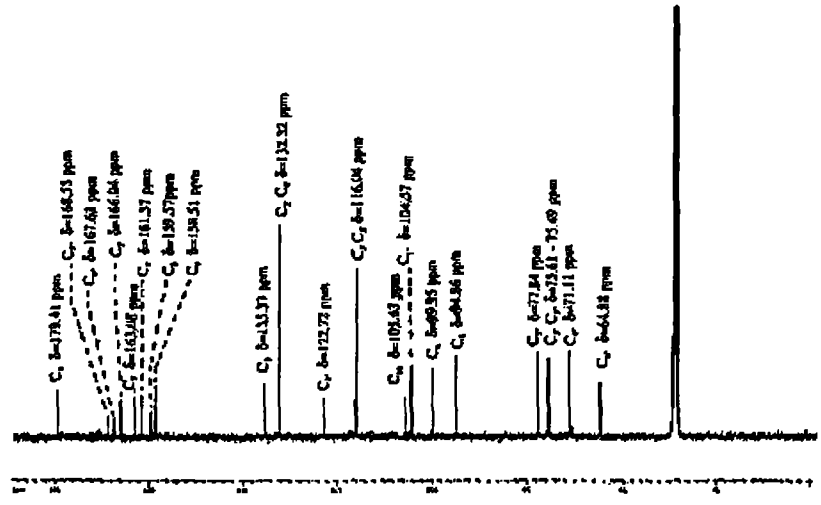

Figure 2. 13C-NMR spectrum of Kaempferol-3-O-(6″-O-malonyl)-ß-D-glucopyranoside

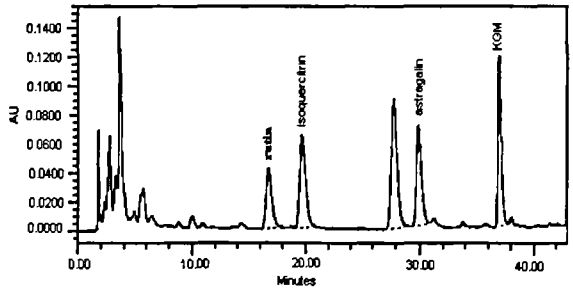

Figure 3. Chromatographic profile of *Ribes nigrum* leaves extract

Table 1. Validation data

LINEARITY	Isoquercitrin			Astragalin			KGM		
Standard									
slope	60691650			60751175			9331724		
intercept	- 39645			- 43497			- 6809		
r	0.9999			0.9999			0.9999		
Leaves extract									
slope	1220765			1264955			1809690		
intercept	-24485			-32614			-30839		
r	0.9997			0.9998			0.9999		
PRECISION	day 1	day 2	day 3	day 1	day 2	day 3	day 1	day 2	day 3
Intra-day precision									
Mean	2274299	2271889	2291938	2294724	2275129	2320529	3364052	3349072	3275314
C.V. (%)_	0.7	1.8	2.1	0.9	2.1	1.9	1.2	2.25	0.60
Accuracy									
recovery	101.0	101.7	100.9	101.1	101.6	100.64	101.1	101.5	100.5
C.V. (%)	1.06	1.30	0.60	1.03	1.43	0.53	1.05	1.45	0.35
Inter-day precision									
Mean	2279376			2296794			3329480		
C.V. (%)	1.69			1.92			1.85		
Accuracy									
recovery	101.2			101.1			101		
C.V. (%)	0.35			0.38			0.4		