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Flavonol glycosides from whole cottonseed by-product

Anna Lisa Piccinelli [a], Attilio Veneziano [a], Siro Passi [b], Francesco De Simone [a], Luca Rastrelli [a,*]

[a] Università, degli Studi di Salerno, Dipartimento di Scienze Farmaceutiche, Via Ponte don Melillo, 84084 Fisciano (SA), Italy
[b] Istituto Dermopatico dell'Immacolata (IDI, IRCCS), Via Monti di Creta, 104 Rome, Italy

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Abstract

Cottonseeds are fed to high-producing dairy cows as a source of fat and highly-digestible fibre. Seven flavonol glycosides have been identified from whole cottonseed by-product. Their structures were established as quercetin 3-*O*-{β-D-apiofuranosyl-(1 → 2)-[α-L-rhamnopyranosyl-(1 → 6)]-β-D-glucopyranoside} (**1**), kaempferol 3-*O*-{β-D-apiofuranosyl-(1 → 2)-[α-L-rhamnopyranosyl-(1 → 6)]-β-D-glucopyranoside} (**2**), quercetin 3-*O*-[β-D-apiofuranosyl-(1 → 2)-β-D-glucopyranoside] (**3**), quercetin 3-*O*-β-D-glucopyranoside (**4**), kaempferol 3-*O*-[α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranoside] (**5**), quercetin 3-*O*-[α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranoside] (**6**), and kaempferol 3-*O*-α-L-rhamnopyranoside (**7**). Gallic acid (**8**) and 3,4-dihydroxybenzoic acid (**9**) were also found. All structures were elucidated by ESI-MS and NMR spectroscopic methods. Total polyphenols were assayed by the Folin–Ciocalteu method.

Keywords: Whole cottonseed; By-products; Flavonol glycosides; 1D and 2D NMR; Feedstuff

1. Introduction

Agricultural processing yields many by-products that have significant feeding value for livestock. Because of their increasing market value as feed ingredients, many producers of such materials now consider them to be "co-products" rather than "by-products." Agricultural co-products that are sometimes available at attractive prices include soy hulls, wheat midds, whole cottonseed, wet and dry corn gluten feed, wet and dried brewers grains, dried distillers grains, poultry litter and many others.

Whole cottonseed (WCS) is the unprocessed and unadulterated oilseed which has been separated from cotton fibre. Delinted cottonseed is the unprocessed and unadulterated oilseed which has been separated from cotton fibre with less than 5% retained lint. Cottonseeds are fed to high-producing dairy cows as a source of fat and highly-digestible fibre. They are also used as a forage replacer. WCS is an excellent source of energy, protein, and effective fibre WCS dry matter is high in fat (200 g/kg), crude protein (CP, 230 g/kg) and neutral detergent fibre (NDF, 440 g/kg); this is reflected in its high energy content (9.2 MJ of net energy for lactation, NEL). The CP:NEL ratio (about 1 g CP to 40 kJ NEL) makes WCS a favourable supplement which meets the combined energy and CP requirements for high-producing dairy cows (National Research Council, 1989). The addition of WCS to the diet of lactating cows during hot weather significantly increased milk yield, milk fat content and yield, and blood plasma triglyceride, cholesterol and phospholipid concentrations (Belibasakis & Tsirgogianni, 1995).

Secondary constituents in whole cottonseed have been studied because some components, including terpenoid phytoalexins (Stipanovic, Bell, & Howell, 1975), have been blamed for anti-nutritive or toxic effects when the seeds were tested as animal feed (Gambill & Humphrey, 1993; Skutches, Herman, & Smith, 1974). Gossypol is the main anti-nutrient limiting the use of cottonseed in monogastric animals and humans, where it acts by reducing the oxygen-carrying capacity of the blood and results in shortness of breath and of edema of the lungs (Alford, Liepa, & Vanbe-

* Corresponding author. Tel.: +39 89 964356; fax: +39 89 962828.
E-mail address: rastrelli@unisa.it (L. Rastrelli).

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ber, 1996). The US Food and Drug Administration sets a limit for free gossypol at 450 mg/kg in human food products and ingredients, and the FAO and WHO set the maximum limitation at 12,000 mg/kg of total gossypol (Lusas & Jividen, 1987).

Information about other phenolic compounds, such as flavonoids, in WCS is scarce and only recently did Zhang, Yang, Zhao, Luan, and Ke (2001) find that glandless cotton seed contains five flavonol glycosides, including a new apiosyl derivative. Flavonoids have been recognized as active principles, showing structure-dependent physiological activity (antioxidant, cancer-preventing, and antimicrobial) (Packer, Hiramatsu, & Yoshikawa, 1999), and their occurrence in food and feed is highly desirable. Owing to the importance of whole cottonseed as a feedstuff, for its high fat and protein contents, we have undertaken a systematic investigation of its secondary metabolites. Particular emphasis was placed on flavonoids that have been shown to possess a range of biological activity which could be beneficial to animal health and potentially could improve flavour and shelf life of farm products (Aerts, Barry, & McNabb, 1999; Simpson & Uri, 1956). Phenolic constituents, in particular, have been shown to be effective free radical-scavengers and antioxidants which are central to the maintenance of homeostasis in biological systems (Ames, Shigenaga, & Hagen, 1993; Torel, Cillard, & Cillard, 1986). Proanthocyanidins and flavonoids have been reported to exhibit antimicrobial (Kabuki et al., 2000; Marwan & Nagel, 1986; Stavric, 1994) and anti-inflammatory activities (Kakegawa et al., 1985; Shoskes, 1998).

As these phenolic compounds are increasingly being regarded as contributing to animal health and productivity, it is important that considerations of the nutritive value of forages should include, not only the primary metabolites, but also the phenolic compounds which they contain.

2. Materials and methods

2.1. Plant material

Whole cottonseed is a by-product of cotton production and acreage is expanding in the north of Italy. Whole cottonseed (WCS) was furnished by Cereal Comm Feed Company (Brescia, Italy).

2.2. Spectroscopic apparatus

UV spectra were recorded on a Shimadzu UV-2101PC, UV–Vis scanning spectrophotometer. A Bruker DRX-600 NMR spectrometer, operating at 599.19 MHz for ^{1}H and at 150.86 MHz for ^{13}C, using the UXNMR software package, was used for NMR experiments; chemical shifts are expressed in δ (parts per million) referred to the solvent peaks δ_H 3.34 and δ_C 49.0 for CD_3OD; coupling constants, J, are in Hertz. DEPT ^{13}C, 1D-TOCSY, ^{1}H–^{1}H DQF-COSY, ^{1}H–^{13}C HSQC, and HMBC NMR experiments were carried out using conventional pulse sequences, as described in the literature. Electrospray ionization mass spectrometry (ESI-MS) was performed using a Finnigan LC-Q Advantage instrument from Thermoquest (San Jose, CA) equipped with Excalibur software. Samples were dissolved in MeOH and infused in the ESI source by using a syringe pump; the flow rate was 3 μl/min. The capillary voltage was 5 V, the spray voltage 5 kV, and the tube lens offset 35 V. The capillary temperature was 220 °C and the data were acquired in the MS1 and MS/MS scanning modes. The scan range was m/z 150–900 and for the MS/MS scanning mode, the percentage of collision energy was 30%. GC analyses were performed using a Chrompack (Middelburg, The Netherlands) model 9001 gas chromatograph with a data-handling system and FID.

2.3. Extraction and isolation procedure of compounds 1–9

The dried and powdered WCS (405 g) was defatted with hexane and $CHCl_3$ and then extracted with MeOH to give 19 g of residue. The MeOH extract was partitioned between *n*-BuOH and H_2O to afford a *n*-BuOH soluble portion (5.8 g) which was chromatographed twice on a Sephadex LH 20 CC (Pharmacia, Uppsala, Sweden) (1 m × 3 cm i.d.) column with flow rate of 0.5 ml/min; 90 fractions of 8 ml were collected. After TLC analysis (Si-gel, *n*-BuOH–AcOH–H_2O 65:15:25, $CHCl_3$–MeOH–H_2O 70:30:3), fractions with similar R_f were combined, giving four major fractions (I–IV) which were further purified by HPLC on a Waters 590 series pumping system equipped with a Waters R401 refractive index detector, a Waters μ-Bondapak C-18, 10 μm (7.8 × 300 mm, flow rate 2.5 ml/min) column and a U6K injector. Fraction II (450 mg) was purified using MeOH–H_2O (40:60) as the eluent, to yield pure flavonol glycosides **1** (30.0 mg, $R_t = 11$ min), **2** (19.0 mg, $R_t = 12$ min), **3** (42.0 mg, $R_t = 18$ min), **4** (28.1 mg, $R_t = 21$ min), **5** (41.3 mg, $R_t = 22$ min), **6** (22.4 mg, $R_t = 28$ min), **7** (29.0 mg, $R_t = 31$ min). Fraction III (205 mg) was purified using MeOH–H_2O (30:70) to yield pure compounds **8** (101.5 mg, $R_t = 10.1$ min) and **9** (78.0 mg, $R_t = 11.8$ min).

2.4. Acid hydrolysis of compounds 1 and 2

A solution of each compound (3.0 mg) in 6% aqueous HCl (3.5 ml) was refluxed for 2 h. The reaction mixture was diluted with H_2O and then extracted with EtOAc. The resulting aglycones were identified by their ^{1}H NMR spectra.

2.5. Methanolysis of compounds 1 and 2

Compounds (1.0 mg of each) were heated in a sealed vial for 24 h at 80 °C in 2% MeOH–HCl (2 ml). After MeOH and HCl distillation in a N_2 stream, Ag_2CO_3 and MeOH were added until CO_2 production stopped. The centrifugate was dried over P_2O_5. The resulting monosaccharides were treated with TRISIL-Z (Pierce) and analysed by GC.

Retention times were identical to those of the authentic trimethylsilyl-sugars.

2.6. Quercetin 3-O-{β-D-apiofuranosyl-(1 → 2)- [α-L-rhamnopyranosyl-(1 → 6)]-β-D-glucopyranoside} (*1*)

Yellow amorphous solid. UV λ_{max} (MeOH): 265, 286, 347; ^{1}H NMR (CD_3OD), aglycone signals: δ 6.20 (1H, d, $J = 2$ Hz, H-6), 6.40 (1H, d, $J = 2$ Hz, H-8), 6.90 (1H, d, $J = 8.5$ Hz, H-5′), 7.62 (1H, dd, $J = 8.5$, 2 Hz, H-6′), 7.64 (1H, d, $J = 2$ Hz, H-2′); ^{13}C NMR (CD_3OD): ppm 158.9 (C-2), 132.2 (C-3), 179.3 (C-4), 163.1 (C-5), 99.8 (C-6), 165.7 (C-7), 94.8 (C-8), 158.4 (C-9), 105.8 (C-10), 123.1 (C-1′), 116.2 (C-2′), 145.8 (C-3′), 149.5 (C-4′), 116.8 (C-5′), 123.5 (C-6′), sugar signals see Tables 1 and 2 (below). ESI-MS *m/z* 741 $[M - H]^-$; MS/MS *m/z* 609 $[(M - H) - 132]^-$, 595 $[(M - H) - 146]^-$, 463 $[(M - H) - 146 - 132]^-$, 301 $[(M - H) - 146 - 132 - 162]^-$.

2.7. Kaempferol 3-O-{β-D-apiofuranosyl-(1 → 2)-[α-L-rhamnopyranosyl-(1 → 6)]- β-D-glucopyranoside} (*2*)

Yellow amorphous solid. UV λ_{max} (MeOH): 266, 286, 348; ^{1}H NMR (CD_3OD), aglycone signals: δ 6.25 (1H, d, $J = 2$ Hz, H-6), 6.44 (1H, d, $J = 2$ Hz, H-8), 6.90 (1H, d, $J = 8.5$ Hz, H-3′, H-5′), 8.12 (1H, d, $J = 8.5$ Hz, H-2′, H-6′), sugar signals: δ 1.11 (3H, d, $J = 6$ Hz, Me–Rha), 4.51 (1H, s, H-l of rhamnose), 5.43 (1H, d, $J = 7.5$ Hz, H-l of glucose), 5.50 (1H, d, $J = 1.5$ Hz, H-l of apiose); ^{13}C NMR (CD_3OD): ppm 158.9 (C-2), 132.2 (C-3), 179.3 (C-4), 163.1 (C-5), 99.8 (C-6), 165.7 (C-7), 94.8 (C-8), 158.4 (C-9), 105.8 (C-10), 123.1 (C-1′), 116.2 (C-2′), 145.8 (C-3′), 149.5 (C-4′), 116.8 (C-5′), 123.5 (C-6′), sugar signals see Table 2. ESI-MS *m/z* 725 $[M - H]^-$; MS/MS *m/z* 593 $[(M - H) - 132]^-$, 579 $[(M - H) - 146]^-$, 447 $[(M - H) - 146 - 132]^-$, 285 $[(M - H) - 146 - 132 - 162]^-$.

2.8. Quercetin 3-O-[β-D-apiofuranosyl-(1 → 2)-β-D-glucopyranoside] (*3*)

Yellow amorphous solid. UV λ_{max} (MeOH): 265, 290, 346; ^{1}H NMR (CD_3OD), aglycone signals: δ 6.20 (1H, d, $J = 2$ Hz, H-6), 6.40 (1H, d, $J = 2$ Hz, H-8), 6.90 (1H, d, $J = 8.5$ Hz, H-5′), 7.62 (1H, dd, $J = 8.5$, 2 Hz, H-6′), 7.64 (1H, d, $J = 2$ Hz, H-2′), sugar signals: 5.43 (1H, d, $J = 7.5$ Hz, H-l of glucose), 5.50 (1H, d, $J = 1.5$ Hz, H-l of apiose); ^{13}C NMR (CD_3OD), aglycone signals: ppm 158.9 (C-2), 132.2 (C-3), 179.3 (C-4), 163.1 (C-5), 99.8 (C-6), 165.7 (C-7), 94.8 (C-8), 158.4 (C-9), 105.8 (C-10), 123.1 (C-1′), 116.2 (C-2′), 145.8 (C-3′), 149.5 (C-4′), 116.8 (C-5′), 123.5 (C-6′), sugar signals see Table 2. ESI-MS *m/z* 595 $[M - H]^-$, MS/MS *m/z* 463 $[(M - H) - 132]^-$, 301 $[(M - H) - 132 - 162]^-$.

Table 1
^{1}H NMR[a] data for the oligosaccharide moiety of **1** in CD_3OD

Proton	Glucose	Apiose	Rhamnose
H-l	5.55, d, $J = 7.5$	5.48, d, $J = 1.5$	4.52, d, $J = 1.5$
H-2	3.67, dd, $J = 7.5$, 9.5	4.07, d, $J = 1.5$	3.65, dd, $J = 3.5$, 1.5
H-3	3.80, t, $J = 9.5$		3.25, dd, $J = 9.5$, 3.5
Ha-4	3.64, t, $J = 9.5$	3.82, d, $J = 10.0$	3.23, t, $J = 9.5$
Hb-4		4.05, d, $J = 10.0$	
Ha-5	3.60, m	3.61, s	3.42, dq, $J = 9.5$, 6.2
Hb-5		3.61, s	
Ha-6	3.69, dd, $J = 12.0$, 3.5		1.10, d, $J = 6.0$
Hb-6	3.58, dd, $J = 12.0$, 5.0		

[a] ^{1}H–^{1}H coupling constants (Hz) in the sugar spin–spin were measured from TOCSY and COSY spectra.

Table 2
^{13}C NMR chemical shift assignments (δ in CD_3OD) of sugar moieties of compounds **1–3**[a]

Carbon[b]	DEPT	**1**	**2**	**3**
Glu-1	CH	102.4	102.3	102.2
-2	CH	75.7	75.6	75.8
-3	CH	78.7	78.8	78.6
-4	CH	72.3	71.9	71.2
-5	CH	78.0	78.1	78.7
-6	CH_2	68.4	68.5	62.3
Rha -1	CH	100.8	100.9	
-2	CH	71.2	72.1	
-3	CH	72.1	72.3	
-4	CH	72.3	72.4	
-5	CH	69.7	69.7	
-6	CH_3	17.8	17.9	
Api -1	CH	110.5	110.7	110.5
-2	CH	77.1	76.7	77.0
-3	C	80.1	80.2	80.4
-4	CH_2	75.4	75.2	75.4
-5	CH_2	66.5	66.2	66.7

[a] Assignments confirmed by 1D TOCSY and 2D COSY, HSQC, HMBC experiments.
[b] Glu = β-D-glucopyranosyl, Rha = α-L-rhamnopyranosyl, Api = β-D-apiofuranosyl.

2.9. Quercetin 3-O-β-D-glucopyranoside (*4*)

Yellow amorphous solid. ESIMS *m/z* 463 $[M - H]^{-1}$, 301 $[(M - H) - 162]^-$. ^{1}H and ^{13}C NMR data were consistent with those previously reported (Agrawal, 1989).

2.10. Kaempferol 3-O-[α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranoside] (*5*)

Yellow amorphous solid. ESMS *m/z* 593 $[M - H]^-$, MS/MS *m/z* 447 $[(M - H) - 146]^-$, 285 $[(M - H) - 146-162]^-$. ^{1}H and ^{13}C NMR data were consistent with those previously reported (Agrawal, 1989).

2.11. Quercetin 3-O-[α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranoside] (*6*)

Yellow amorphous solid. ESIMS *m/z* 609 $[M - H]^-$, MS/MS *m/z* 463 $[(M - H) - 146]^-$, 301 $[(M - H) - 146 - 162]^-$. ^{1}H and ^{13}C NMR data were consistent with those previously reported (Agrawal, 1989).

2.12. Kaempferol 3-O-α-L-rhamnopyranoside (7)

Yellow amorphous solid. ESIMS *m/z* 431 $[M - H]^-$, 285 $[(M - H) - 146]^-$. ^1H and ^{13}C NMR data were consistent with those previously reported (Agrawal, 1989).

2.13. Gallic acid (8)

White amorphous solid. ESIMS *m/z* 169 $[M - H]^-$. ^1H and ^{13}C NMR data were consistent with those previously reported (Nuñez et al., 2002).

2.14. 3,4-Dihydroxybenzoic acid (9)

Yellow amorphous solid; ESIMS *m/z* 153 $[M - H]^-$. ^1H and ^{13}C NMR data were consistent with those previously reported (Nuñez et al., 2002).

2.15. Total polyphenols assay

Estimation of the global polyphenol content in the extracts was performed according to the Folin–Ciocalteu method. Whole Cotton Seed was extracted with acetone/water (7:3), employing a Dionex Accelerated Solvent Extractor ASE-200. A portion (1.15 mg) of this extract was dissolved in MeOH (2 ml), and the extract was diluted 10-fold with water. Folin–Ciocalteu reagent (0.5 ml; Merck) was added to the diluted solutions (0.5 ml), followed by 0.5 ml of a 100 g/l solution of Na_2CO_3. The absorbance was measured at 720 nm (Shimadzu UV-2101 spectrophotometer) with a blank sample (water plus reagents) in the reference cell. Quantification was obtained by reporting the absorbances relative to the calibration curve of quercetin used as standard phenol.

3. Results and discussion

Whole cotton seed was extracted, successively, with hexane, $CHCl_3$ and MeOH. The MeOH extract was partitioned between *n*-BuOH and H_2O to afford an *n*-BuOH-soluble portion which was subjected to chromatography on Sephadex LH-20 and HPLC. Nine phenolic compounds were isolated (Fig. 1): quercetin 3-*O*-{β-D-apiofuranosyl-(1 → 2)-[α-L- rhamnopyranosyl-(1 → 6)]-β-D-glucopyranoside} (**1**), kaempferol 3-*O*-{β-D-apiofuranosyl-(1 → 2)-[α-L-rhamnopyranosyl-(1 → 6)]-β-D-glucopyranoside} (**2**), quercetin 3-*O*-[β-D-apiofuranosyl-(1 → 2)-β-D-glucopyranoside] (**3**), quercetin 3-*O*-β-D-glucopyranoside (**4**), kaempferol 3-*O*-[α-L-rhamnopyranosyl-(1 → 6)-β-D- glucopyranoside] (**5**), quercetin 3-*O*-[α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranoside] (**6**), kaempferol 3-*O*-α-L-rhamnopyranoside (**7**), gallic acid (**8**) and 3,4-dihydroxybenzoic acid (**9**). The structures and molecular of formulae of compounds **1–9** were determined from their ESI-MS spectra, as well as from ID and 2D ^1H and ^{13}C NMR data.

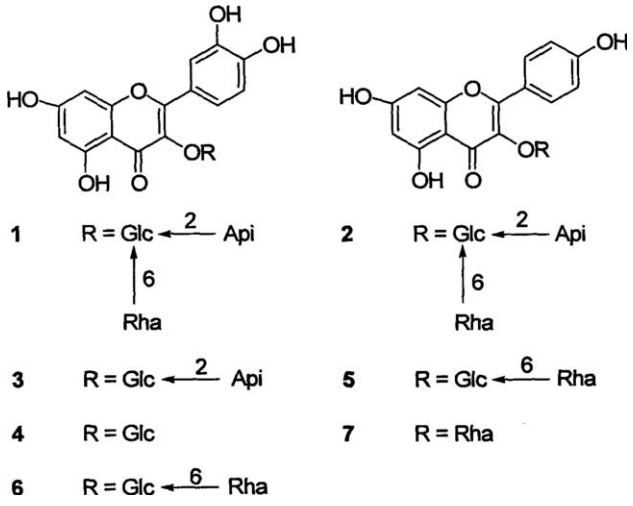

Fig. 1. Flavonol glycosides **1–7** isolated from whole cotton seeds.

Acid hydrolysis of **1** and **2** released, respectively, quercetin and kaempferol, identified by ^1H and ^{13}C NMR spectroscopy. The gas chromatographic analysis of the methanolysis products showed the presence, for both compounds, of glucose, apiose and rhamnose, in the ratio 1:1:1. The ESI-MS (negative ion) of **1** gave, as base peak, the $[M - H]^-$ ion at *m/z* 741, whereas compound **2** showed an $[M - H]^-$ ion 725, 16 mass units lower than that of **1**. From the mass and ^{13}C NMR and ^{13}C DEPT NMR data, the molecular formulae $C_{32}H_{38}O_{20}$ and $C_{32}H_{38}O_{19}$ were deduced for compounds **1** and **2**, respectively.

The complete structures of **1** and **2** were elucidated by ID and 2D NMR experiments at 600 MHz. The ^1H NMR spectrum of **1** showed, for the aromatic part, two signals at δ 6.20 and 6.40 (both d, $J = 2$ Hz) which could be assigned to H-6 and H-8, respectively, and signals at δ 7.64 (d, $J = 2$ Hz), 6.90 (d, $J = 8.5$ Hz) and 7.62 (dd, $J = 8.5$, 2 Hz) could be ascribed to H-2′, H-5′ and H-6′, respectively. The ^{13}C NMR shifts of the aglycone part of **1** corresponds to the shifts for quercetin, the only significant differences being those for C-2 and C-3. These shifts are analogous to those reported when the 3-hydroxy group is glycosylated in a flavonol glycoside (Agrawal, 1989). Three anomeric protons were easily identified in the spectra of **1** at δ 5.55 (d, $J = 7.5$ Hz), 5.48 (d, $J = 1.5$ Hz) 4.52 (d, $J = 1.5$ Hz) and correlated with carbons at δ 102.4, 100.8, and 110.5, respectively. From the assigned aglycone and sugar values, it was apparent that a trisaccharide unit was attached to C-3 of the aglycone. The structure of the sugar chain was assigned by a combination of 1D-TOCSY, 2D DFQ-COSY, HSQC, and HMBC experiments. Starting from the anomeric protons of each sugar unit, all of the hydrogens within each spin system could be identified using a combination of 1D-TOCSY and COSY experiments (Table 1). The assignments of all proton resonances for the sugar moieties allowed us to assign the resonances of the linked carbon atoms by HSQC experiment (Table 2). Information about the sequence of the oligosaccharide chain was

deduced from HMBC experiments. Key correlation peaks were observed between the anomeric proton of the glucose (δ 5.55) and the C-3 of the quercetin (δ 132.2), between the anomeric proton signal of the rhamnose (δ 4.52) and the C-6 of glucose (δ 68.4) and between the anomeric proton of the apiose (δ 5.48) and the C-2 of the glucose (δ 75.7). From these considerations, the structure as quercetin, 3-*O*-{β-D-apiofuranosyl-(1 → 2)-[α-L-rhamnopyranosyl-(1 → 6)]-β-D-glucopyranoside} (Fig. 1) was assigned to **1**. This compound was reported previously by our research group as a constituent of *Chenopodium pallidicaule* seeds (Rastrelli, Saturnine, Schettino, & Dini, 1995).

The ^{1}H NMR spectrum of **2** displayed signals for two *meta*-coupled protons at δ 6.25 (d, $J = 2$ Hz, H-6) and 6.44 (d, $J = 2$ Hz, H-8) and also for an *ortho*-coupled system at δ 8.12 (d, $J = 8.5$ Hz, H-2′ and H-6′) and 6.90 (d, $J = 8.5$ Hz, H-3′ and H-5′), indicating a kaempferol derivative. The other signals in the ^{1}H, ^{13}C, and ^{13}C DEPT NMR data were superimposable on those of **1**. These data suggested that the structure of **2** is kaempferol 3-*O*-{β-D-apiofuranosyl-(1 → 2)-*O*-[α-L-rhamnopyranosyl-(1 → 6)]-β-D-glucopyranoside} (Fig. 1). Compound **2** was reported for the first time in 2001 in a journal written in Chinese (Zhang et al., 2001); thus, we report the NMR data here.

Compound **3** presented almost superimposable ^{1}H and ^{13}C NMR spectra on those of compound **1**. The main differences were the absence of the rhamnose signals at δ 4.52 in the ^{1}H NMR spectra, and the absence of the corresponding signals in the ^{13}C NMR spectra. From these considerations, the structure of quercetin 3-*O*-[β-D-apiofuranosyl-(1 → 2)-β-D-glucopyranoside] was assigned to **3**.

Quercetin 3-*O*-β-glucopyranoside (**4**) was also characterized by its NMR data and ESI-MS spectra (Agrawal, 1989).

Compounds **5** and **6** were identified as kaemferol 3-rutinoside and quercetin 3-rutinoside, respectively, by UV spectral analysis and ^{1}H and ^{13}C NMR spectroscopy. The substitution pattern on glucose was deduced by comparison of the ^{13}C NMR chemical shifts with the data for quercetin 3-*O*-β-glucopyranoside (**4**). The downfield shifts of 7.1 ppm for C-6 indicated a glycosidic linkage at C-6 for both **5** and **6**.

The ^{1}H NMR spectrum for compound **7** showed a signal corresponding to a rhamnose residue at a typical shift δ 1.18 ppm ($J = 6.2$ Hz). The large singlet, at δ 4.56 ppm ($J = 1.5$ Hz), indicated an α-configuration of the anomeric proton of the sugar (H-1′). Identification as kaempferol 3-*O*-α-rhamnopyranoside was confirmed by ESI-MS data and by comparison of the ^{1}H NMR shifts and coupling constants with the literature data (Agrawal, 1989).

Compound **8** was identified as gallic acid from its ^{1}H NMR spectrum and further confirmed by comparison of its chromatographic behaviour with that of an authentic sample.

Compound **9** showed spectroscopic data identical with those of 3,4-dihydroxybenzoic acid.

WCS contains fractions that are rich in flavonol glycosides, including the 7 compounds identified. They contain the aglycones of quercetin and kaempferol, and carbohydrate moieties, (as mono-, di- and trisaccharides), linked to the aglycones the C-3 position. The quantitative total polyphenol content (1.90 g/kg) obtained from the Folin–Ciocalteu assays, generally considered as the method of choice to estimate total phenol contents in plant extracts (Scalbert, 1992), and the structural variability, are interesting due to the alimentary and taxonomic properties ascribed to flavonol glycosides. Flavonol apiosides **1** and **2** are rare natural products.

4. Conclusion

Whole cottonseed is a by-product of cotton production and acreage is expanding in the north of Italy. Whole cottonseed can be processed for its oil content. An increasing proportion has been used as feed in recent years. Cottonseed is high in total digestible nutrients (94%) and crude protein (23%) and is a good feed for cattle. The range of phenolic compounds present in WCS provides a valuable database for chemotaxonomic investigation as well as for possible nutritional studies on the impact of diet on animal health and productivity. Recent studies have shown that many plant phenols possess a range of bioactivities, including antimutagenic and free radical-scavenging activities (Hanasaki, Ogawa, & Fukui, 1994; Jovanovic, Steenken, Simic, & Hara, 1998) which, although not very significant to ruminants, due to their restricted life spans, could be important to consumers of farm products. In view of these properties, it would be reasonable to expect that ruminants fed on fodders rich in polyphenols may retain some of these desirable polyphenols in their milk or flesh which could benefit consumers (Vinson, Hao, Su, & Zubik, 1998). In addition, the presence of such antioxidants and free radical-scavengers could improve the quality and shelf life of farm products by their ability to inhibit lipid peroxidation.

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