Characterization of flavonoid glycosides from rapeseed bee pollen using a combination of chromatography, spectrometry and nuclear magnetic resonance with a step-wise separation strategy

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To identify the structures of flavonoid glycosides in bee pollen collected from rapeseed plants (\textit{Brassica napus} L.), we utilised an approach that combined liquid chromatography–diode array detector–electrospray ionization–mass spectrometry (LC–DAD–ESI–MS) and nuclear magnetic resonance (NMR) technology with a step-wise separation strategy. We identified four constituents of high purity in rape bee pollen samples: (1) quercetin-3-\textit{O}\textendash\textbeta\textendash\textD-glucosyl-(2\rightarrow1)-\textbeta\textendash\textD-glucoside, (2) kaempferol-3, 4\textprime\textbeta\textendash\textD-glucoside, (3) 5, 7, 4\textprime\textbeta\textendash\textD-trihydroxy-3\textprime\textbeta\textendash\textD-methoxyflavone-3-\textbeta\textendash\textD-sophoroside and (4) kaempferol-3-\textbeta\textendash\textD-glucosyl-(2\rightarrow1)-\textbeta\textendash\textD-glucoside. This study will also provide useful reference standards for qualification and quantification of four flavonoid glycosides in natural products.

Keywords: bee pollen; step-wise separation strategy; semi-preparative HPLC; LC–DAD–ESI–MS; NMR; flavonoid glycosides

1. Introduction

The bee pollen of the important crop rapeseed consists of pollen mixed with plant nectars and bee secretions (Eraslan et al. \textit{2009}). Bee pollen has long been a popular dietary supplement
(Nicolson & Human 2013) and additive used in the cosmetics, food and medicine industry. Rape bee pollen is used widely in traditional Chinese medicine to prevent atherosclerosis and treat prostatic hyperplasia (Murakami et al. 2008). Flavonoids and their glycosides found in bee pollen are a large group of secondary plant metabolites that have a wide range of biological effects, including anti-oxidant, anti-inflammatory, anti-allergen, anti-ulcer, antibiotic and anti-carcinogenic properties. There is a therefore growing interest in the isolation and identification of the flavonoid constituents of rape bee pollen, in order to lay the foundation for study of their biological activities. Some of them have been isolated from rape bee pollen and other plants and identified via MS, NMR spectroscopic analysis and chemical evidence (Serra Bonvehí et al. 2001; Cho et al. 2004; Guo 2009; Maruyama et al. 2010; Hao et al. 2012). LC–DAD–MS and NMR have proven to be powerful methods for the identification of flavonoid structure by providing molecular weight and fragmentation information (DeStefano & Kirkland 1975; Stahl 2003; Negri et al. 2013; Xie et al. 2011, 2013).

The purpose of this work was to develop a convenient and efficient method for the reliable separation and identification of flavonoid glycosides compounds from rape pollen. Firstly, flavonoid glycoside compounds were successively isolated from rape pollen using octadecylsilyl (ODS) chromatography and semi-preparative high-performance liquid chromatography (semi-prep HPLC). Secondly, the purities and chemical structures of the isolated compounds were elucidated by DAD, LC–ESI–MS and NMR. Flavonoid glycosides were identified by comparing their HPLC retention times, ultra-violet (UV) absorption and MS fragmentation characteristics. Further, $^1$H and $^{13}$C NMR was utilised to support the HPLC–DAD–ESI–MS identification, in order to discover new flavonoid glycosides whose reference standards were not commercially available, or that were previously unknown to occur in rape pollen.

2. Results and discussion

2.1. Extraction and isolation of flavonoid glycosides from rape bee pollen

The overall fractionation procedure that we used is presented in Figure S1. It has also been reported that the maximum extraction efficiency of phenolic acids from rape bee pollen was obtained using ultrasonic-assisted extraction with 80% ethanol as the extraction solvent (Yang et al. 2010). Three fractions (B$_1$, B$_2$ and B$_3$) from A$_2$ were obtained under the isocratic elution condition (14% aqueous acetonitrile). Then four fractions (C$_1$, C$_2$, C$_3$ and C$_4$) from B$_2$ were obtained at retention times between 27 and 43 min (Figure S2). The individual UV absorption maxima of fractions C$_1$ to C$_4$ were 255, 265, 270 and 265 nm, and all of the spectra had a slight shoulder peak at 339–353 nm (Figure S3). Moreover, flavonoids exhibit maximum absorbance in the vicinity of 280 and 360 nm in the UV region. That confirmed that fractions C$_1$ to C$_4$ were flavonoid compounds, results that are consistent with those reported in the literature (Zhou et al. 2014).

2.2. Elucidation of flavonoid glycoside structures

2.2.1. Fraction C$_1$

Based on Figure S4(A), we concluded that fraction C$_1$ contained two hexose bases and a quercetin group. From the $^1$H and $^{13}$C-NMR spectrum (Figure S5(A) and (B)), the presence of these structural elements enabled us to conclude that fraction C$_1$ is a flavonoid glycoside, defined as quercetin-3-O-β-D-glucosyl-(2→1)-β-glucoside. Its structural formula C$_1$ is shown in Figure S6(A).

2.2.2. Fraction C$_2$

Based on Figure S4(B), we concluded that fraction C$_2$ contained two hexose bases and a kaempferol moiety. From the $^1$H and $^{13}$C-NMR spectrum (Figure S7(A) and (B)), these
structural elements indicated that fraction C_2 is a flavonoid glycoside, defined as kaempferol-3, 4'-di-O-β-d-glucoside. Its structural formula is shown in Figure S6(B).

2.2.3. Fraction C_3
Based on Figure S4(C), we concluded that fraction C_3 contained two hexose bases and an isorhamnetin moiety. From the ^1H and ^13C-NMR spectrum (Figure S8 (A) and (B)), these structural elements indicated that fraction C_3 is a flavonoid glycoside, defined as 5, 7, 4'-trihydroxy-3'-methoxyflavone-3-O-β-d-sophoroside. Its structural formula is shown in Figure S6 (C).

2.2.4. Fraction C_4
Based on Figure S4(D), we concluded that fraction C_4 contained two hexose bases and a kaempferol moiety. From the ^1H and ^13C-NMR spectrum (Figure S9 (A) and (B)), these structural elements showed that fraction C_4 is a flavonoid glycoside, defined as kaempferol-3-O-β-d-glucosyl-(2→l)-β-d-glucoside. Its structural formula is shown in Figure S6 (D).

2.2.5. Hepatoprotective activity assay
The hepatoprotective activities of the isolated compounds against CCl_4-induced human L02 cells were determined according to previously reported methods (Zhou et al. 2013). IC_{50} is defined as the concentration of target analytes that reduces cellular activities by 50% in comparison with untreated control cultures. Quercetin-3-O-β-d-glucosyl-(2→l)-β-glucoside showed the hepatoprotective effect with IC_{50} value of 132.64 ± 4.1 μM, whereas three other compounds have no such activity.

3. Conclusions
In conclusion, we isolated and identified four flavonoid glycosides including quercetin-3-O-β-d-glucosyl-(2→l)-β-glucoside, kaempferol-3, 4'-di-O-β-d-glucoside, 5, 7, 4'-trihydroxy-3'-methoxyflavone-3-O-β-d-sophoroside, and kaempferol-3-O-β-d-glucosyl-(2→l)-β-d-glucoside in bee pollen.

Supplementary material
Experimental details relating to this paper are available online, alongside Tables S1–S4 and Figures S1–S9.

Disclosure statement
No potential conflict of interest was reported by the authors.

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Note
1. Jinhui Zhou and Liping Sun contributed equally to this work.
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