Food Chemistry 218 (2017) 600–609

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Efficient identification of flavones, flavanones and their glycosides in routine analysis via off-line combination of sensitive NMR and HPLC experiments

Martina Blunder [a,c,*], Andreas Orthaber [b,d], Rudolf Bauer [a], Franz Bucar [a], Olaf Kunert [b]

[a] Institute of Pharmaceutical Sciences, Department of Pharmacognosy, University of Graz, Universitätsplatz 4, 8010 Graz, Austria
[b] Institute of Pharmaceutical Sciences, Department of Pharmaceutical Chemistry, University of Graz, Universitätsplatz 1, 8010 Graz, Austria
[c] Department of Neuroscience, Biomedical Center, Uppsala University, Box 593, 75124 Uppsala, Sweden
[d] Department of Chemistry, Ångström Laboratories, Uppsala University, Box 523, 75120 Uppsala, Sweden

ARTICLE INFO

Article history:
Received 28 February 2016
Received in revised form 11 September 2016
Accepted 12 September 2016
Available online 13 September 2016

Keywords:
NMR
Flavones
Flavanones
HPLC
MS
Data base

ABSTRACT

We present a standardized, straightforward and efficient approach applicable in routine analysis of flavonoids combining sensitive NMR and HPLC experiments. The determination of the relative configuration of sugar moieties usually requires the acquisition of ^{13}C NMR shift values. We use a combination of HPLC and sensitive NMR experiments (1D-proton, 2D-HSQC) for the unique identification of known flavones, flavanones, flavonols and their glycosides. Owing to their broad range of polarity, we developed HPLC and UHPLC methods (H_2O/MeOH/MeCN/HCOOH) which we applied and validated by analyzing 46 common flavones and flavanones and exemplified for four plant extracts. A searchable data base is provided with full data comprising complete proton and carbon resonance assignments, expansions of HSQC-spectra, HPLC parameters (retention time, relative retention factor), UV/Vis and mass spectral data of all compounds, which enables a rapid identification and routine analysis of flavones and flavanones from plant extracts and other products in nutrition and food chemistry.

1. Introduction

Flavonoids are regarded as important secondary metabolites in food and medicinal plants due to their various health promoting and therapeutic activities. They prove to be radical scavengers, exert anti-inflammatory activity by inhibiting enzymes of the arachidonate pathway (COX-1,-2, 5-LOX) and other mediators of inflammation (elastase, histamine, etc.); furthermore their cancer preventive, anti-edematous, anti-hemorrhagic, spasmolytic and diuretic activities have been shown (Middleton, Kandaswami, & Theoharides, 2000; Nijveldt et al., 2001). Moreover, it has also been suggested that flavonoids decrease the risk of coronary heart disease (Mojzisova & Kuchta, 2001) and flavonoid rich diets have beneficial effects against acquired insulin resistance (Chudnovskiy et al., 2014) and improve cognition in older adults (Brickman et al., 2014). Flavonoids are essential ingredients in plant-based foods and their occurrence in vegetables, fruits, beverages and other related food products is well documented (Andersen & Markham, 2006; Jandera et al., 2005). Due to their wide distribution in the plant kingdom and their significance in quality control of foods, botanical dietary supplements as well as herbal medicinal products, rapid identification of known flavonoids and their glycosides is a routine task in many laboratories.

Undoubtedly, flavonoids and their glycosides form a large number of isomeric compounds which usually cannot be identified by a single, fast and widely available technique like HPLC/PDA (photodiode array) or HPLC/MS. Clearly, the development of LC–MS^n techniques resulted in a significant improvement of structural analysis of flavonoids. Recently, multistage mass spectrometric techniques for analysis of flavonoid glycosides have been thoroughly reviewed by Hossain, Rai, Brunton, Martin-Diana, and Barry-Ryan (2010) and Vukics and Guttman (2010). However, no information on the stereochemistry of the glycan part of the flavonoid glycosides can be obtained by mass spectrometric methods. In contrast, NMR spectroscopy allows the unambiguous identification of flavonoids but requires time consuming experiments especially as flavonoid identification is for historic reasons based on recording of intrinsically insensitive 1D-carbon experiments.

* Corresponding author at: Department of Neuroscience, Biomedical Center, Uppsala University, Sweden, Brain Institute, Federal University of Rio Grande do Norte, UFRN, Natal, Brazil.
E-mail addresses: martina.blunder@uni-graz.at (M. Blunder), andreas.orthaber@kemi.uu.se (A. Orthaber), rudolf.bauer@uni-graz.at (R. Bauer), franz.bucar@uni-graz.at (F. Bucar), olaf.kunert@uni-graz.at (O. Kunert).

http://dx.doi.org/10.1016/j.foodchem.2016.09.077
0308-8146/

Experience shows that in most cases a combination of different spectroscopic techniques is required for identification of flavonoids. Recent trends in coupling NMR with chromatographic techniques, like e.g. HPLC (Christophoridou, Dais, Tseng, & Spraul, 2005; Dai et al., 2009; Lambert, Hansen, Sairafianpour, & Jaroszewski, 2005; Tatsis et al., 2007) or capillary isotachophoresis (cITP) (Kautz et al., 2001; Wolters, Jayawickrama, & Sweedler, 2005) led to a significant improvement in identification of natural products. Comprehensive reviews on on-line and off-line hyphenated NMR methods are given by Jaroszewski (2005a, 2005b) and Seger & Sturm (2007). Diffusion-order spectroscopy (DOSY) is a powerful tool in the direct analysis of mixtures, which has been successfully applied in flavonoid analytics. (Rodrigues et al., 2009) Recently, various additives, like polyethylenglycol (PEG) or sodium dodecyl sulfate (SDS) have been used to improve resolution in these DOSY measurements of flavonoid containing mixtures. (Kavakka, Parviainen, Wähälä, Kilpeläinen, & Heikkinen, 2010) Compared to direct HPLC NMR hyphenation off-line techniques usually provide higher quality of NMR spectra and allow by multiple trapping an increase in analyte amounts. Despite the significant gain of instrumental and experimental sensitivity NMR based approaches still rely on time-consuming NMR resonance assignments in case of flavonoid identifications. Exemplified by 46 flavonoids, we present a combined experimental approach which benefits from both the advantages of HPLC (UHPLC) and NMR: These flavones and flavanones are fully characterized by the combination of HPLC retention time, UV, ESI-MS and proton or HSQC NMR data. The 2D HSQC experiment can be acquired in short time – compared to directly detected ^{13}C spectra – especially for milligram and sub-milligram amounts of flavonoids. Under these conditions acquisition times of HSQC experiments are reduced by more than a factor of 10 compared to direct detection of ^{13}C spectra. Despite the relative high percentage of quaternary carbon atoms in flavonoids, the multiplicity-edited HSQC experiment often highlights important structural features of the compound under investigation. By correlating proton and carbon chemical shifts it defines fingerprint regions, e.g. in which it is easy to determine the number of attached saccharide units or to distinguish different fusion patterns in saccharide side chains. While epimeric compounds often give almost identical retention times, which prevents direct identification by HPLC coupled to a PDA or MS detector, these structural differences lead to characteristic differences of chemical shift values in NMR spectra. Therefore, a straightforward identification can be accomplished by comparison of measured ^{1}H and ^{1}H–^{13}C HSQC correlations with standardized reference data.

Moreover, the chromatographic and NMR data – shift values and graphical representation of complete ^{1}H and ^{13}C spectra and expansions of 2D HSQC spectra – are provided in a data base and are supplemented by complete tabulated proton and carbon NMR resonance assignments, UV data, and ESI-MS fragmentation data. All spectral data and depicted spectra (^{1}H, ^{13}C, relevant expansions of HSQC spectra) are given with an interface to a Microsoft Access data base which can easily be expanded and modified by the user. This allows both, identification of the compound via retention time and ^{1}H or HSQC spectra and also enables a straightforward search for other spectral data.

2. Experimental

2.1. Materials

Compounds **24**, **29**, **30**, **32** and **33** were taken from our collection of reference materials, **19**, **25**, **38** were purchased from Sigma Chemical Co. (St. Louis, MO, USA), **13** and **26** were obtained from Extrasynthese (Genay, France), all other flavonoids were obtained from Carl Roth (Karlsruhe, Germany). The solvents used for chromatography were of HPLC grade, bought either from Roth or Sigma Aldrich (Steinheim, Germany). Formic acid 98% originated from Merck (Darmstadt, Germany). Deuterated pyridine was purchased from Euriso-Top (Saint-Aubin Cedex, France) and supplemented with 0.1% (v/v) tetramethylsilane from Roth.

Plant extracts of elderflowers (*Sambucus nigra* L.) and heather (*Calluna vulgaris* (L.) Hull) were prepared by accelerated solvent extraction using 80% (v/v) methanol as described previously (Rieger, Mueller, Guttenberger, & Bucar, 2008). In addition, lemon (*Citrus limon* (L.) Osbeck) and bitter orange (*Citrus x aurantium* L.) peels were extracted with methanol (30 min under reflux).

2.2. NMR spectroscopy

All NMR data of the 46 reference compounds were recorded in pyridine-d_5 at 298 K. This solvent allows experiments with both the unsubstituted aglycons and highly methylated or glycosylated flavonoids. Sample quantities ranged from 1 mg to 10 mg, dissolved in 720 µl pyridine-d_5, for naringenin (**38**) a data set was recorded in DMSO-d_6, too. All experiments were performed on a Varian UnityInova 600 MHz spectrometer using NMR experiments from the Varian pulse sequence library. Experimental parameters for the used hardware are listed in Seebacher, Simic, Weis, Saf, and Kunert (2003), the referencing of ^{13}C and ^{1}H resonances was done with TMS as internal standard. The HSQC was optimized for a 130 Hz coupling and the HMBC for a 7 Hz coupling. The proton NMR shift values were given with two decimal places, carbon shift values with one decimal place.

2.3. HPLC analysis

Compounds were chromatographed on two different HPLC systems, i.e. Agilent 1100 series system, Degaser G1311 A, Quat Pump G1311, Autosampler G1313 A, Colcom G1316 A, DAD 1315 B) and Merck Lachrome (pump L7100, autosampler L-2700, photodiode-array detector L7455). Two different columns were used: Zorbax SB C-18, 150 × 2.1 mm, 3.5 µm (Agilent) and LiChrospher 100 RP-18, 125 × 4 mm, 5 µm (Merck). Injection volumes were 5 µl on both columns. Flavonoids were analyzed using a linear gradient, 0.1% formic acid in water (v/v) (A) 0.1% formic acid in acetonitrile (v/v) (B), 0.1% formic acid in methanol (v/v) (C); starting with 95% A, 2% B, 3% C and finalizing at 40 min. with 43% B and 57% C, respectively (flow rate, 0.4 ml/min (Zorbax); 1.2 ml/min (LiChrospher); column temperature, 30 °C; PDA: 220–500 nm).

2.4. HPLC-PDA-ESI-MS analysis

Analysis was performed by an UHPLC method on an UltiMate 3000 RS HPLC system coupled to a LTQ XL mass spectrometer (Thermo Scientific) using a Kinetex C18 column (100 × 2.1 mm, 1.7 µm; Phenomenex), column temperature, 30 °C; injection volume, 3 µl. Eluents consisted of 0.1% formic acid in water (v/v) (A) and 0.1% formic acid in methanol – acetonitrile 4:3 (v/v). The linear gradient started with 95% A and 5% B, finalizing with 100% B in 21.33 min, returning to initial conditions in 0.54 min and equilibrating for 6.4 min; flow rate, 500 µl/min; PDA range from 190 to 500 nm. Gradient conditions for UHPLC analysis were adapted from those used with the Zorbax column by a gradient method calculator (Thermo Scientific). Mass spectrometric detection: electrospray ionization (negative mode), capillary temperature 330 °C; source heater temperature 250 °C; sheath gas flow, 50 arbitrary units (a.u.); auxiliary gas flow, 10 a.u.; capillary voltage, −16.00 V; source voltage, 3.0 kV; source current, 100 µA; normalized collision energy, 35%; mass range, 50–2000 amu.