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

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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.

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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.

2.5. SPE-trapping and elution with pyridine-d_5

A sample of the non-polar flavonoid naringenin (**38**) was prepared by dissolving 2 mg in 2 ml methanol. One half of this solution was dried and re-dissolved in pyridine-d_5 (720 μL) serving as NMR calibration reference. The other half was diluted with 4 ml water and trapped on a pre-conditioned SPE-RP-C18 column (100 mg, Isolute, Biotage), blow-dried with nitrogen and eluted with pyridine-d_5 (1500 μL). The eluate was then dried and re-dissolved in pyridine-d_5 (720 μL). Peak intensities of this sample were compared with the NMR reference and indicated an elution efficiency of >95% with pyridine.

3. Results

In this work we provide a facile route to flavonoid routine analysis through a combination of HPLC retention times and proton and carbon resonances by sensitive NMR experiments, such as 1D proton and 2D HSQC spectra. We analyzed 46 flavonoids by means of HPLC/PDA, HPLC/MS and NMR spectroscopy. Our full data include UV and mass spectral data, (relative) HPLC retention times, fully assigned proton and carbon resonances, electronic copies of proton and carbon spectra, and relevant expansions of HSQC and HMBC spectra. In order to facilitate handling of the collected data, they are organized in a MS Access data base with a user-friendly and intuitive search mask.

Portions of NMR samples with sub-milligram amounts of flavonoids can usually not directly be used in bio-assays as the final concentration of the organic solvent must not exceed 1%, therefore, prior to the use in bio-assays removal of the solvent is necessary. For that reason, pyridine was chosen for our NMR experiments since it can be removed more easily from the sample compared to DMSO and allows facile use of the compounds in bio-assays after NMR data acquisition. Moreover, pyridine is an excellent solvent for polar as well as non-polar substances and hence allows identical experimental conditions for all types of flavonoids.

Several previous series of ^{13}C data sets recorded in DMSO exist, (Markham & Ternai, 1976; Markham, Ternai, Stanley, Geiger, & Mabry, 1978) nevertheless the high experimental temperature in these studies is not possible on many modern routine NMR hardware without damaging the shim system. In modern NMR devices maximal temperatures of 70 °C should not be exceeded in order to prevent partial destruction of the shim coil system. Though, higher sample temperatures can be realized when external cooling of the shim coils is provided. Thus, this limitation prevents a simple supplementation of those existing carbon reference data with proton NMR shift values under identical experimental conditions. Recent data sets collected in MeOH-d_4, (Moco, Tseng, Spraul, Chen, & Vervoort, 2006) on the other hand, exclude identical experimental conditions for very lipophilic flavonoids. Therefore all data have been rerecorded under standardized conditions in our study. Many of the previous assignments of ^{13}C resonances were based on the use of incremental systems and contained a lot of uncertainties or even assignment errors. Hence, for all compounds a complete set of 1D ^{1}H, ^{13}C and gradient-selected 2D $^{1}H,^{13}C$-HSQC, $^{1}H,^{13}C$-HMBC and $^{1}H,^{1}H$-DQF-COSY experiments was recorded and all assignments were done *de novo*.

The presented selection of 46 compounds covers a diverse set of flavonoids and comprises 36 flavones (including 21 flavonoles) and 10 flavanones (Fig. 1) which include aglyca, mono-, di and triglycosylated flavonoids and two C-glycosides. Commonly used trivial names and semi-systematic names for all compounds are listed in Table 1.

In the course of this study we developed and evaluated a suitable reversed phase (RP)-HPLC method for the analysis, identification and isolation of flavonoids. In addition, an UHPLC method was elaborated. In contrast to established binary systems (Andersen & Markham, 2006) we used a ternary solvent system (H_2O/MeOH/MeCN) and formic acid (0.1%). The latter was used as a constant additive in order to improve reproducibility. This ternary solvent system yields an excellent separation for a range of compounds with different polarity, providing a significantly enhanced resolution for both flavonoid glycosides and flavonoid aglyca, hence, justifying the increased analytical runtime compared to binary solvent methods. For the UHPLC method a fixed mixture of methanol and acetonitrile (4:3, v/v) had to be applied, resulting in similar retention values compared to the gradient systems for the Zorbax and LiChrospher columns. Using a similar UHPLC method a reduction of analysis time by about 47% could be achieved.

The HPLC experiments were performed on three different HPLC units (Agilent, Merck, Thermo/Dionex) and three different RP-C18 columns (Zorbax, LiChrospher, Kinetex), respectively. Method validations indicated a good *intra-* and *inter* day reproducibility, which was specifically tested for the aglycon acacetin (**3**) and the mono-glycoside naringenin-7-glucoside (**42**), as representatives for non-polar and polar flavonoids, respectively. In order to make a sound comparison of retention times we also calculated the retention factor (k′), which provides a flow- and column dimension-independent index. These compounds gave retention factors k′ with maximum deviations of ±0.03 for *intra* and for uninterrupted *inter* day variability. A maximum variation in k′ by ±0.09 was observed for long term *inter* day reproducibility. However, a relative retention factor, referring to the abundant and cheaply available rutin (**35**), is given. This makes the given data machine independent and hence allows good comparison with other systems and also significantly improves long term *inter* day comparisons. Fig. 2 illustrates the different retention behavior for the employed columns. While at low k′ values both columns show similar behavior, we observe larger deviations for k′ values beyond ca. 20. This effects mostly the non-glycosylated flavonoids resulting in longer retention times for the Zorbax© column.

With respect to the determined *inter* day reproducibility in uninterrupted and interrupted runs of ±0.03 and ±0.09, a reliable differentiation of two compounds in *inter* day HPLC runs is possible, if their k′ differ by more than 0.06 and 0.18 ($1\sigma = 95\%$ confidence interval), respectively. Using an internal standard and k′ values relative to rutin gives excellent reproducibility.

3.1. Unambiguous identification using NMR data

As a first indication, the analysis of UV spectra might serve well for the distinction of compounds with very similar k′ values, because different classes of flavonoids, i.e. flavones (including flavonols), flavanones, exhibit characteristic UV maxima (Markham & Mabry, 1975). However, in the presented series of 46 flavonoids we observed only very few cases, where two compounds with similar retention times belonged to different classes of flavonoids (e.g. the flavonol **21** and flavone **6**). In many cases of isomeric glycosylated flavonoids neither HPLC, nor UV, nor MS nor a combination thereof provided a sufficient set of data for unambiguous identification. Therefore, in many cases the acquisition of NMR data is essential in flavonoid analysis.

In our study, a large set of 1D and 2D NMR data (^{1}H, ^{13}C, HSQC, HMBC, DQF-COSY) was acquired. Complete and correct assignments of proton and carbon resonance shift values were done for each of the 46 flavonoids. For unambiguous assignment of carbon C-6 and C-8 we used additional available NMR data of narigenin recorded in DMSO-d_6 to confirm these assignments recorded in pyridine-d_5 with HMBC correlations between 5-OH and C-6.

For a simple and rapid data handling we created an interface for MS Access data base, which enables the user to display formulas,

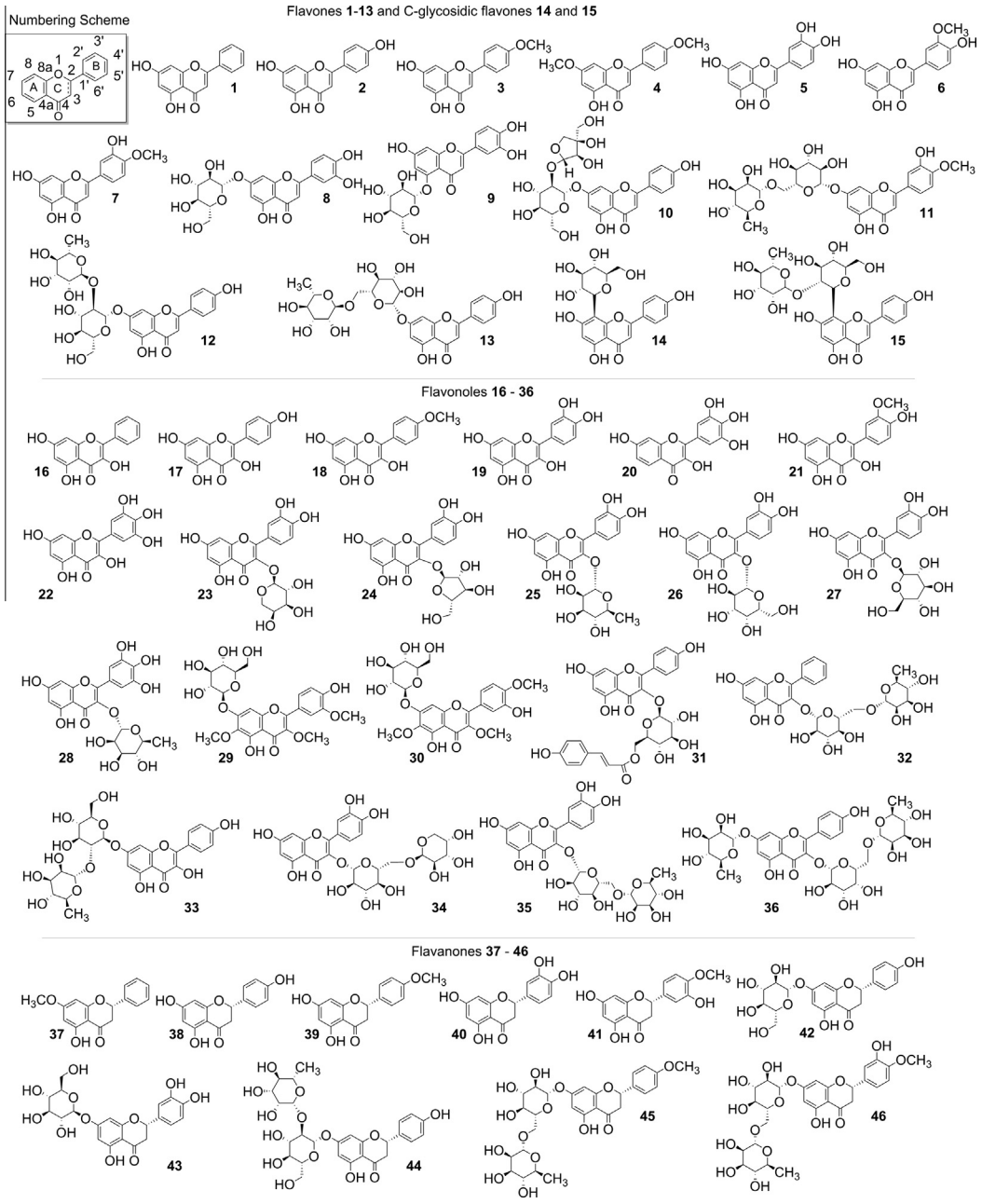

Fig. 1. Structures of flavonoids and their glycosides investigated in this study: Schematic drawings of flavones **1**–**13**, C-glycosidic flavones **14** and **15**, flavonoles **16**–**36** and flavanones **37**–**46** and general numbering scheme of flavonoids.

common names, CAS numbers, molecular weights, relevant NMR spectroscopic information like assigned proton and carbon shift values, graphical representations of the proton and carbon 1D spectra and characteristic expansions of 2D HSQC spectra. Furthermore, HPLC retention factors k′ for three different RP-columns (two for HPLC, one for UHPLC), retention factors k′ relative to rutin (**35**), corresponding UV spectral data as well as ESI-MS data of molecular ions and fragmentations are provided in a searchable database.

As a first step in the efficient identification of a known compound its retention time (Table 2) is determined by HPLC with one of the described standard methods. This reduces the number of potential flavonoids to a manageable quantity. Subsequently, the proton 1D or 2D HSQC NMR spectra are recorded (in pyridine-d_5, 298 K) to resolve any ambiguity due to similar retention times and/or MS and UV data.

With the limited number of remaining potential flavonoids at the given retention time, the recorded NMR data can be easily compared with their reference data. For this purpose the data base offers principally two options:

The numerical NMR shift values of the unknown compound are compared with the data of the potential matches extracted from the data base in tabulated form.

Expansion of the 2D HSQC spectrum or the proton spectrum of the unknown compound is visually compared with the corresponding spectra of the potential matches in the database.

Both methods should yield a correct identification and are usually done within short time. There is no need for time consuming acquisition of a full NMR data set (including carbon spectra) or, above all, complete assignments of HMBC correlations. Throughout this work a consistent numbering of core atoms is followed facilitating easy comparison with our reference data (Fig. 1, inset).

In addition to the above described rapid identification of flavonoids, the data base allows the user to supplement additional information for the provided compounds and extend the data base

Table 1
Trivial names and semi-systematic names for the investigated flavonoids.

No	MW	Trivial name	Systematic name
Flavones			
1	254.06	Chrysin	5,7-Dihydroxyflavone
2	270.24	Apigenin	5,7,4′-Trihydroxyflavone
3	284.26	Acacetin	5,7-Dihydroxy-4′-methoxyflavone
4	298.29	Apigenin-7,4′-dimethylether	5-Hydroxy-7,4′-dimethoxyflavone
5	286.24	Luteolin	5,7,3′,4′-Tetrahydroxyflavone
6	300.26	Chrysoeriol	5,7,4′-Trihydroxy-3′-methoxyflavone
7	300.26	Diosmetin	5,7,3′-Trihydroxy-4′-methoxyflavone
8	448.38	Luteolin-7-*O*-glucoside	Luteolin-7-*O*-*β*-D-glucopyranoside
9	448.38	Luteolin-5-*O*-glucoside	Luteolin-5-*O*-*β*-D-glucopyranoside
10	564.49	Apiin	Apigenin-7-*O*-*β*-D-apiofuranosyl (1→2)-*β*-D-glucopyranoside
11	608.54	Diosmin	Diosmetin-7-*O*-*β*-D-rutinoside
12	578.52	Rhoifolin	Apigenin-7-*O*-*β*-D-neohesperidoside
13	578.52	Isorhoifolin	Apigenin-7-*O*-*β*-D-rutinoside
C-Glycosyl flavones			
14	432.10	Vitexin	Apigenin-8-*C*-*β*-D-glucoside
15	578.16	Vitexin-2′-rhamnoside	Apigenin-8-*C*-*α*-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranoside
Flavonoles			
16	270.24	Galangin	3,5,7-Trihydroxyflavone
17	286.24	Kaempferol	3,5,7,4′-Tetrahydroxyflavone
18	326.00	Kaempferide	3,5,7-Trihydroxy-4′-methoxyflavone
19	302.24	Quercetin	3,5,7,3′,4′-Pentahydroxyflavone
20	302.24	Robinetin	3,7,3′,4′,5′-Pentahydroxyflavone
21	326.26	Isorhamnetin	3,5,7,4′-Tetrahydroxy-3′-methoxyflavone
22	318.04	Myricetin	3,5,7,3′,4′,5′-Hexahydroxyflavone
23	434.08	Guaijaverin	Quercetin-3-*O*-*α*-L-arabinopyranoside
24	434.08	Avicularin	Quercetin-3-*O*-*α*-L-arabinofuranoside
25	462.12	Quercitrin	Quercetin-3-*O*-*α*-L-rhamnopyranoside
26	464.38	Hyperosid	Quercetin-3-*O*-*β*-D-galactopyranoside
27	464.38	Isoquercitrin	Quercetin-3-*O*-*β*-D-glucopyranoside
28	464.38	Myricitrin	Myricetin-3-*O*-*α*-L-rhamnopyranoside
29	522.46	Jacein	5,7,4′-Trihydroxy-3,6,3′-trimethoxy-flavone-7-*O*-*β*-D-glucoside
30	522.46	Centaurein	5,7,3′-Trihydroxy-3,6,4′-trimethoxy-flavone-7-*O*-*β*-D-glucoside
31	594.53	Tiliroside	Kaempferol-3-*O*-*β*-D-(6′-*O*-(*E*)-p-coumaroyl)glucopyranoside
32	578.53	Galangin-3-rutinoside	Galangin-3-*O*-*β*-D-rutinoside
33	594.52	Kaempferol-7-neohesperoside	Kaempferol-7-*O*-*β*-D-neohesperidoside
34	596.49	Quercetin-3-arabino-glucoside	Quercetin-3-*O*-*α*-arabinopyranosyl-(1′→6′)-*β*-glucopyranoside
35	610.52	Rutin	Quercetin-3-*O*-*β*-D-rutinoside
36	740.66	Robinin	Kaempferol-3-*O*-*α*-L-rhamnopyranosyl-(1→6)-β-D-galacto-pyranosyl-7-*O*-*α*-L-rhamnopyranoside
Flavanones			
37	270.09	7-Methylpinocembrin	5-Hydroxy-7-methoxyflavanone
38	272.25	Naringenin	5,7,4′-Trihydroxyflavanone
39	286.28	Isoakuranetin	5,7-Dihydroxy-4′-methoxyflavanone
40	288.55	Eriodictyol	5,7,3′,4′-Tetrahydroxyflavanone
41	302.28	Hesperetin	5,7,3′-Trihydroxy-4′-methoxyflavanone
42	434.39	Naringenin-7-glucoside	Naringenin-7-*O*-*β*-D-glucopyranoside
43	450.39	Eriodictyol-7-glucoside	Eriodictyol-7-*O*-*β*-D-glucopyranoside
44	580.53	Naringin	Naringenin-7-*O*-*β*-D-neohesperidoside
45	594.56	Didymin	Isoakuranetin-7-*O*-*β*-D-rutinoside
46	610.56	Hesperidin	Hesperetin-7-*O*-*β*-D-rutinoside

with available data of further flavonoids. While this work focuses on the combination of NMR and HPLC, the data base offers the opportunity to create user-defined combined queries, e.g. combination of retention factor and MS or UV, which are more convenient for other tasks. Even in case of unknown flavonoids which are not included in the data set, this collection of spectral data will facilitate identification by combining UV, mass and NMR data and chromatographic behavior in comparison with our reference compounds. Experience shows, that hard-copies of spectral data are still very common in daily lab use. Thus, we provide a predefined form for data sheet print-outs suitable for the creation of a spectra catalog or queries for the compilation of shift values for selected compounds.

In the subsequent section we provide seven illustrative examples for different flavonoids with very similar retention times, which are easily distinguished by means of NMR spectroscopy. Within this section different substitution patterns as well as different numbers and types of saccharide units are compared. Fast and efficient distinction of these pairs is shown on the basis of 1D proton NMR or 2D HSQC correlations, which are readily available within short time.

Isomeric glycosides with similar retention times and different saccharide moieties at the same aglycon position could easily be identified in HSQC. The two epimeric compounds Hyperosid (**26**) and Isoquercitrin (**27**) ($\Delta k' = 0.18$, LiChrospher), differ only in the configuration of the carbon atom C-4 in the saccharide unit (Glcp vs. Galcp). This results in almost identical UV spectra and MS data. Meaningful carbon-proton correlations, however, which can easily be extracted from HSQC spectra, allow distinction of glucose and galactose moieties (Fig. 3, 1a and 1b).

In case of the compounds guaiaverin (**23**) and avicularin (**24**) ($\Delta k' = 0.27$, Kinetex), the same saccharide unit (arabinose) exists either in the pyranose or in the furanose form and is attached to the same aglycon. Again, a differentiation between these isomers is not possible by means of UV or MS data. On the other hand, the ^{13}C chemical shift values of the saccharide units can, once more, easily be extracted from the HSQC experiments serving for simple distinctions of the isomers. In addition, the diastereotopic methylene protons H-5′ in the pyranose form show large chemical shift differences in the HSQC spectrum ($\delta_H = 3.81/4.38$ ppm) as expected. In the furanose form, however, the protons H-5′ form an exocyclic methylene group with small differences of their proton shift values due to rotation of this group ($\delta_H = 4.10/4.15$ ppm).

Sometimes, flavanone gylcosides with a different number of saccharide units show the same retention time. For instance, naringin (**44**) with two saccharide moieties attached to C-7 of the naringenin aglycon and naringenin-7-glucoside (**42**), with only one glucose attached to C-7, are not clearly distinguishable by HPLC with UV-detection ($\Delta k' = 0.08$, LiChrospher). However, the saccharide finger print region of the HSQC NMR spectra clearly show the different numbers of saccharide units in these glycosides and, therefore, allow a straightforward identification, which would also be possible by HPLC-MS. (Fig. 3, 2a and 2b)

Another common structural motif in flavonoids is the presence of either rutinose or neohesperidose moieties attached to the same position of the aglycon, exemplified by isorhoifolin (**13**) and rhoifolin (**12**). While HPLC retention times ($\Delta k' = 0.36$, Kinetex), UV and MS data are similar for both isomeric compounds, the HSQC spectra show clear differences (Fig. 3): The easily accessible carbon shift of the glucose methylene group (C-6′) is low-field shifted in case of the rutinoside ($\delta_C = 67.6$ ppm) due to the glycosidation compared to position C-6′ in the neohesperoside ($\delta_C = 62.2$ ppm). (Fig. 3, 3a and 3b)

In case of the two flavones apiin (**10**) and rhoifolin (**12**) *O*-disaccharide chains are attached to position C-7 of an apigenin aglycon. The terminal saccharid unit is an apiofuranose or a rhamnopyranose, respectively. Despite the obvious chemical difference (pentose vs. hexose) the retention factors are quite similar ($\Delta k' = 0.23$, LiChrospher column). However, in case of rhamnose, a

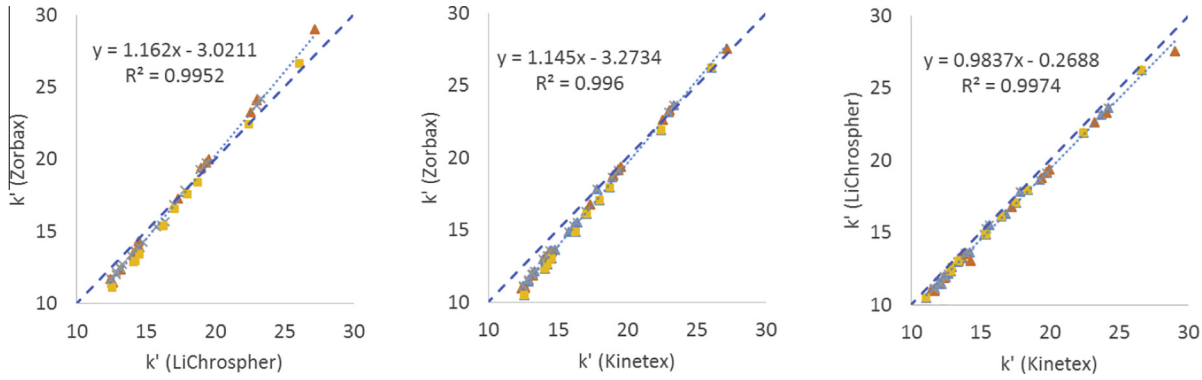

Fig. 2. Comparison of utilized columns in the present study in a k′ vs. k′-plot. Red: Flavone; Green: Flavanoles; Grey: Flavanones. Aglyca are indicated by a black edge. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Retention factors and relative retention factors for the 46 flavonoids determined for two different HPLC columns (Zorbax and LiChrospher) and column (Kinetex) used for UHPLC experiments. Relative retention factors are given with respect to the flavonole rutin (**35**). The flavonoids are ordered by their elution on the Zorbax column.

Comp.	Zorbax©		LiChrospher©		Kinetex©	
	k′	rel. k′	k′	rel. k′	k′	rel. k′
9	12.42	0.94	11.69	0.94	10.94	0.92
34	12.52	0.95	11.62	0.94	11.09	0.93
36	12.52	0.95	11.72	0.94	11.11	0.93
43	12.60	0.96	11.11	0.89	10.49	0.88
14	12.60	0.96	11.46	0.92	10.96	0.92
15	12.65	0.96	11.46	0.92	11.02	0.92
20	12.90	0.98	12.18	0.98	11.45	0.96
28	12.90	0.98	12.00	0.97	11.55	0.97
26	13.13	0.99	12.51	1.01	11.89	1.00
8	13.19	1.00	12.38	1.00	11.85	0.99
35	13.19	1.00	12.41	1.00	11.94	1.00
27	13.31	1.01	12.69	1.02	12.13	1.02
23	13.94	1.06	13.36	1.08	12.96	1.09
42	14.09	1.07	12.89	1.04	12.34	1.03
13	14.12	1.07	13.46	1.08	13.17	1.10
44	14.23	1.08	12.97	1.05	12.60	1.06
33	14.25	1.08	13.62	1.10	13.21	1.11
24	14.25	1.08	13.66	1.10	13.23	1.11
10	14.28	1.08	13.66	1.10	13.26	1.11
25	14.46	1.10	13.95	1.12	13.57	1.14
11	14.48	1.10	14.32	1.15	13.02	1.09
46	14.53	1.10	13.41	1.08	13.02	1.09
12	14.55	1.10	13.89	1.12	13.53	1.13
22	14.80	1.12	14.26	1.15	13.60	1.14
32	15.79	1.20	15.31	1.23	14.89	1.25
29	16.15	1.22	15.41	1.24	15.28	1.28
40	16.29	1.23	15.38	1.24	14.87	1.24
30	16.40	1.24	15.66	1.26	15.53	1.30
19	17.01	1.29	16.85	1.36	16.28	1.36
45	17.08	1.31	16.54	1.33	16.13	1.35
5	17.33	1.31	17.26	1.39	16.77	1.40
31	17.82	1.35	17.89	1.44	17.83	1.49
38	18.01	1.36	17.59	1.42	17.06	1.43
41	18.77	1.42	18.41	1.48	17.94	1.50
17	18.93	1.43	19.28	1.55	18.68	1.56
2	18.97	1.44	19.43	1.57	18.77	1.57
21	19.36	1.47	19.77	1.59	19.13	1.60
6	19.36	1.47	19.77	1.59	19.17	1.61
7	19.52	1.48	19.97	1.61	19.36	1.62
39	22.46	1.70	22.41	1.81	21.94	1.84
1	22.57	1.71	23.26	1.87	22.62	1.89
16	23.00	1.74	23.74	1.91	23.19	1.94
3	23.03	1.75	24.08	1.94	23.30	1.95
18	23.34	1.77	24.23	1.95	23.62	1.98
37	26.09	1.98	26.62	2.14	26.23	2.20
4	27.20	2.06	29.03	2.34	27.55	2.31

6-deoxy mannose, a characteristic duplet of the methyl group is expected around δ_H = 1.85 ppm in the proton spectrum. This resonance, however, is missing in the spectrum of an apiofuranose (Fig. 3, 4a and 4b).

Isomeric *O*-methylated flavonoids like chrysoeriol (**6**) and diosmetin (**7**) often show similar retention times ($\Delta k'$ = 0.16, Zorbax column). However, the NMR shift values of the aromatic protons of the A- and C-ring allow a rapid identification of these

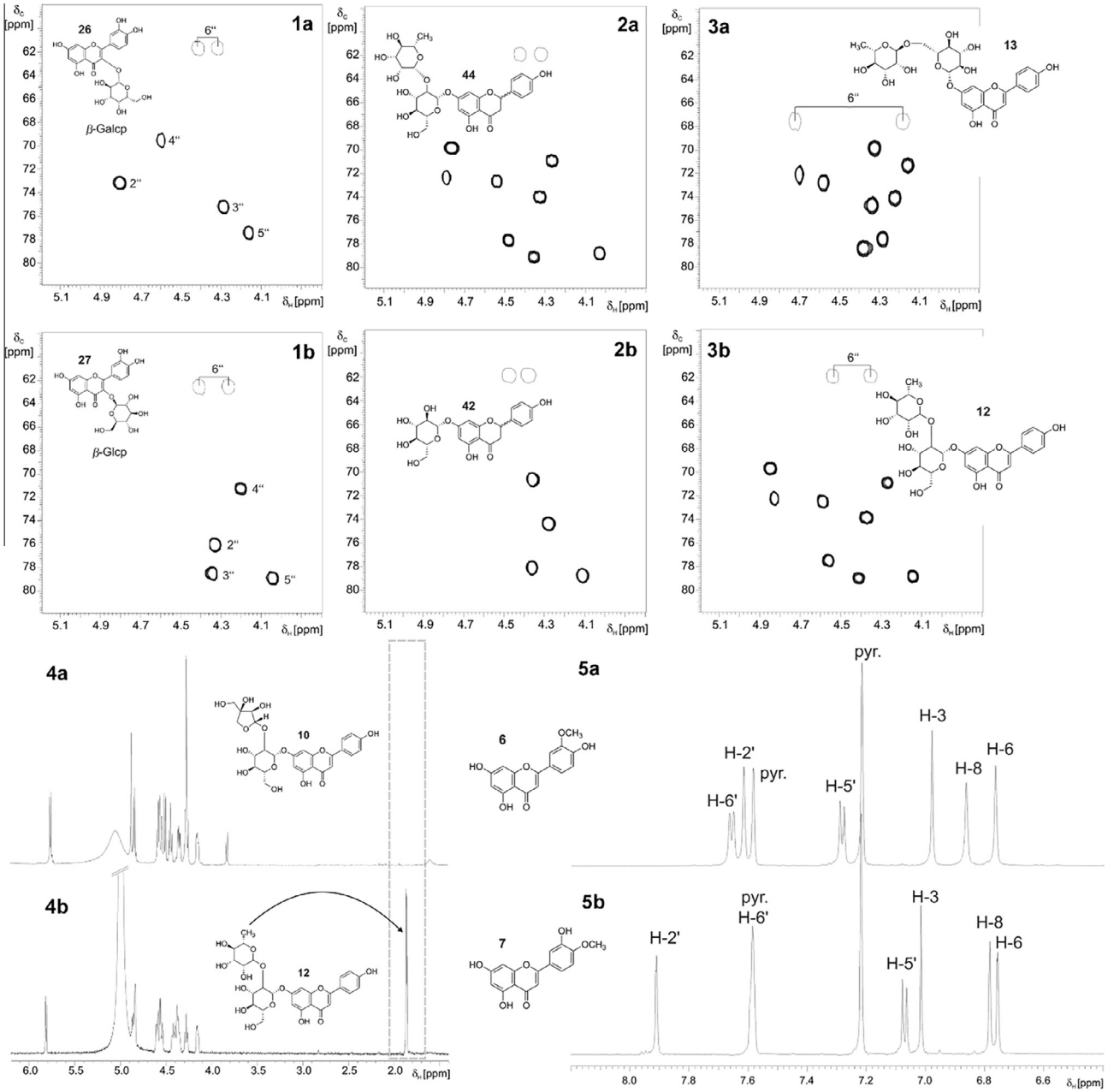

Fig. 3. (1 a + b) Expansion of multiplicity-edited HSQC spectra of **26** and **27**, showing the sugar fingerprint regions. Note the significantly different dispersion of the CH correlations of the two epimeric sugar moieties (galcp vs. glcp). (2 a + b) Expansion of multiplicity-edited HSQC spectra of **44** and **42**, showing the sugar fingerprint regions. The monosaccharide naringenin-7-glucoside (**42**) is easily distinguishable from naringin (**44**). (3 a + b) Expansion of multiplicity-edited HSQC spectra of **13** and **12**, showing the sugar fingerprint regions. Note the significantly different carbon and proton shift values of the C6′-methylene groups of the two glucose moieties. (4 a + b) Expansion of the proton spectra of **10** and **12**, showing the sugar regions. Highlighted is the characteristic resonance of the methyl group for the rhamnose moiety in rhoifolin (**12**). (5 a + b) Expansion of the proton spectra of chrysoeriol (**6**, upper panel) and diosmetin (**7**, lower panel), showing the aromatic regions. In case of **7**, the resonance of H-6′ perfectly overlaps with the solvent resonance in the proton dimension.

compounds. The shift values for H-8, H-2′, H-5′ H-6′ are δ_H = 6.87/7.62/7.28/7.66 ppm of **6**, while the corresponding shift values of **7** are found to be δ_H = 6.78/7.91/7.07/7.58 ppm (Fig. 3, 5a and 5b).

The same is observed in case of the Zorbax resin for the *C*-glycosylated flavones vitexin (**14**) and vitexin-2′-*O*-rhamnoside (**15**). Very similar retention times ($\Delta k'$ = 0.05, Zorbax) but clear differences in proton or HSQC NMR spectra are observed, which allow easy assignments.

Although HPLC analysis in combination with either MS or PDA detection provides valuable information for the identification of known flavonoids, it is obvious that in many cases additional information is needed. Hence acquisition of relevant – and promptly available – NMR data (i.e. proton and HSQC spectra) usually leads to an unambiguous identification.

3.2. Application in the analysis of plant extracts

In order to assess the applicability of the proposed NMR solvent pyridine as eluent in SPE trapping, we trapped the medium-polar naringenin (**38**) on a common SPE-RP-C18 column. Noteworthy, in these trapping/elution experiments pyridine was successfully used for elution. No signs of degradation of the SPE material nor the flavonoid was detected. Observed recovery rates were greater

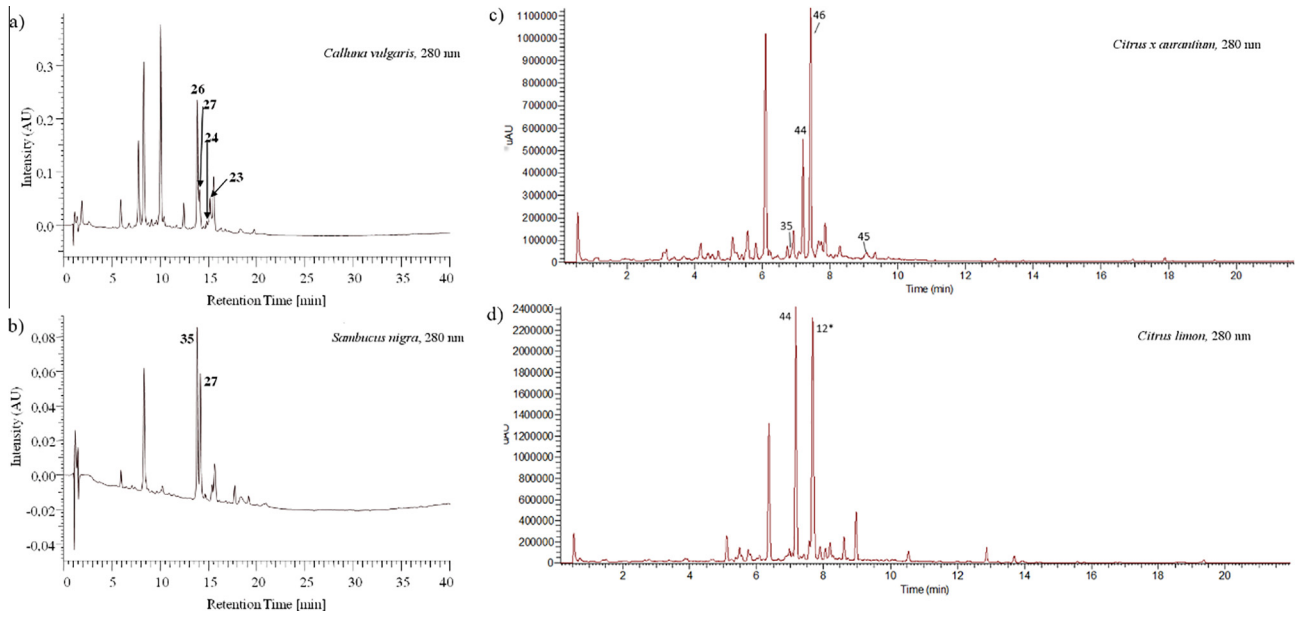

Fig. 4. HPLC and UHPLC chromatographic profiles of *C. vulgaris* (**a**), *S. nigra* (**b**) and *C. x aurantium* (**c**), C. limon (**d**), respectively. The peaks of identified compounds are labeled with the respective compound numbers: rhoifolin (**12**), guaijaverin (**23**), avicularin (**24**), hyperosid (**26**), isoquercitrin (**27**), rutin (**35**), naringin (**44**), hesperidin (**46**). *Co-eluting with neohesperidin (Mencherini et al., 2012).

than 95% indicating that pyridine is an excellent choice for off-line hyphenation of HPLC and NMR.

Hydroalcoholic/methanolic plant extracts usually contain complex mixtures of secondary metabolites of different compound classes including phenolic acid derivatives and flavonoids, making their chromatographic separation challenging. The HPLC systems elaborated in this study are also suitable for analysis of crude plant extracts as illustrated in Fig. 4a + b showing the chromatograms of 80% (v/v) methanolic extracts of elderflowers (*S. nigra*) and heather (*C. vulgaris*). The major flavonoid peaks are sufficiently separated in order to make identification by spectroscopic means possible. Isoquercitrin (**27**) was identified in both extracts, by means of its retention time and characteristic UV and mass spectroscopic data. The absolute retention times of this compound were 14.10 min and 14.15 min in case of the *C. vulgaris* and *S. nigra* extracts, respectively. The retention time of the reference material was found to be 14.11 min showing the good reproducibility of this HPLC method.

The suitability of the developed UHPLC method for flavonoid analysis in crude plant extracts is demonstrated in Fig. 4c + d. The chromatograms show the methanolic extracts from lemon and bitter orange peels which are known to be rich in flavanone and flavone glycosides and aglycones. (Anagnostopoulou, Kefalas, Kokkalou, Assimopoulou, & Papageorgiou, 2005; Mencherini et al., 2012) Naringin could be unequivocally identified in both extracts. Separation of the major *Citrus* flavonoid glycosides was achieved within ca. 9 min.

4. Discussion

In summary, we present an off-line hyphenation of HPLC and sensitive NMR experiments, which provides an efficient and accurate method for the rapid identification of known flavones and flavanones. This approach works in contrast to other, sophisticated ones, e.g. (Donarski, Jones, & Charlton, 2008; March & Brodbelt, 2008) with widely distributed hardware. MS based approaches, despite their simple use, often fail in case of flavonoid glycosides, because the identification of the saccharide types and their relative configuration remains elusive (Park et al., 2008).

HPLC retention times can show some variations depending on several factors differing in various laboratories, but relative values to a specific reference compound are extremely robust. Therefore, a relative retention factor, referring to the abundant and cheaply available rutin (**35**), is given. This makes our given data machine-independent and hence a good comparison with other systems possible. Recently UHPLC has become an important analysis tool also for flavonoid identification significantly decreasing costs and time (Teixeira, Bertoldi, Lajolo, & Hassimotto, 2015; Xu, 2013). The presented HPLC methods display good correlation of retention factors between the three employed columns making it widely applicable.

Essentially, the classical – and still very common – NMR based approach for identification of flavonoids relies on ^{13}C data comparison (Burns, Ellis, & March, 2007). However, as the amount of compounds purified by semi-preparative HPLC is usually smaller than a milligram, the acquisition of 1D carbon spectra should be avoided in routine NMR. Although, hyphenated techniques like HPLC-SPE-NMR are expected to yield a drastically enhanced sensitivity and thus identification of natural products directly from plant extracts might become possible, identification of some compound classes, e.g. flavonoids or coumarins, still remains a challenging and time consuming task (Teixeira et al., 2015) owing to the large number of isomeric structures. Especially for these compound classes reference data recorded under standardized conditions can dramatically reduce the time for resonance assignments needed for identification. Proton and multiplicity-edited HSQC spectra are recorded within significantly shorter time (ca. one order of magnitude), which give sufficient information for fast structure elucidation, especially for flavonoid glycosides (see Fig. 3). Our presented HPLC method was proven to be well suited for the analysis of flavonoid containing plant extracts (see Fig. 4). The large demand for flavonoid identification both in herbal dietary supplements, herbal medicinal products and food quality control will hopefully be a prime application for the method presented herein.

While by far not all of the known flavonoids are included, we provide a representative spectrum of various common flavonoids covering different skeletons, substitution patterns and types and numbers of saccharide units. Consequently, the provided data base can also be helpful for analysis of new compounds by comparing shared structural motifs.

Methods for the prediction of ^{13}C chemical shift values when analyzing spectra of new compounds as published by Burns (Burns et al., 2007) require correctly assigned NMR resonances. Hence our comprehensive data set can provide an excellent basis for prediction tools. Also recent advances in the field of off-line hyphenated NMR methods (Jaroszewski, 2005a, 2005b) could easily be combined with our method. Another benefit is that our reference data strategy can augment NMR-based metabolomics, as shown on the basis of 1D NMR experiments for flavonoid rich preparations of *Ginkgo biloba* (Agnolet, Jaroszewski, Verpoorte, & Staerk, 2010) or on the basis of 2D correlations experiments, which allow multivariate analysis with NMR data (Farag, Mahrous, Lübken, Porzel, & Wessjohann, 2014). The required (qualitative) HMBC correlations can easily be deduced from our fully assigned NMR data sets.

Furthermore, a robust peak picking of proton resonance values can be expected when recent developments in proton homonuclear broadband decoupling are implemented on NMR spectrometers used for routine measurements (Meyer & Zangger, 2013; Sakhaii et al., 2013; Zangger & Sterk, 1997), which will allow automated database searches.

In conclusion, we provide a straightforward and efficient approach towards rapid flavonoid identification by combining simple HPLC and NMR methods facilitating routine analysis of known (and identification of unknown) flavones and flavanones from plant extracts, as well as other products used in nutrition and food chemistry.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.09.077.

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