ELSEVIER

Journal of Chromatography B, 777 (2002) 111–122

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Review

Deuterated phytoestrogen flavonoids and isoflavonoids for quantitation☆

K. Wähälä*, S. Rasku, K. Parikka

Organic Chemistry Laboratory, Department of Chemistry, University of Helsinki, P.O. Box 55, FIN-00014 Helsinki, Finland

Abstract

Isotopically and isomerically pure polydeuterated flavonoids and isoflavonoids have been prepared for quantitation of these compounds in biological matrices. Various deutero-labeling techniques are presented and methods for establishing the isotopical and isomerical purity of deuterated products are discussed.

Keywords: Reviews; Phytoestrogens; Flavonoids; Isoflavonoids

Contents

1. Introduction

Given the potential application of flavonoids and isoflavonoids (Table 1) in the prevention and treatment of a number of serious chronic diseases, there is a strong multidisciplinary interest in the accurate and specific measurement of flavonoids in body

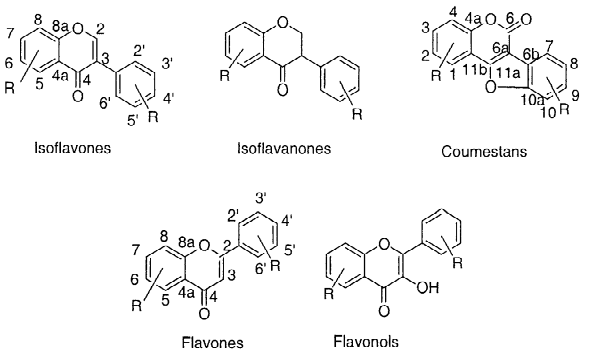
☆This review is based on the PhD thesis of S. Rasku, University of Helsinki (2000) and the MSc thesis of K. Parikka, University of Helsinki (2001).

*Corresponding author. Tel.: +358-9-1915-0356; fax: +358-9-1915-0357.

E-mail address: kristiina.wahala@helsinki.fi (K. Wähälä).

1570-0232/02/$ – see front matter
PII: S1570-0232(02)00096-X

Table 1
Structures, names, and numbering of the flavonoids discussed in this review



	Flavonoid	Substitution
Isoflavone	Daidzein **1**	7,4′-$(OH)_2$
	Formononetin **2**	7-OH, 4′-OCH_3
	Genistein **3**	5,7,4′-$(OH)_3$
	Biochanin A **4**	5,7-$(OH)_2$, 4′-OCH_3
Isoflavanone	Dihydrodaidzein **5**	7,4′-$(OH)_2$
	Dihydrogenistein **6**	5,7,4′-$(OH)_3$
Coumestan	Coumestrol **7**	3,9-$(OH)_2$
Flavone	Apigenin **8**	5,7,4′-$(OH)_3$
	Luteolin **9**	5,7,3′,4′-$(OH)_4$
	2′,3′-Dihydroxyflavone **10**	2′,3′-$(OH)_2$
Flavonol	Fisetin **11**	3,7,3′,4′-$(OH)_4$
	Quercetin **12**	3,5,7,3′,4′-$(OH)_5$

fluids and food samples. The improvement in methods for the quantitation of flavonoids and isoflavonoids and their metabolites has been of great importance in understanding their chemical and biological properties.

There is wide variation in the flavonoid or isoflavonoid contents of plants and food products reported in the literature [1–10] (Table 2). In part this may be due to the different plant varieties and growth conditions, but probably more often to differences in isolation and analytical techniques. For example, the dietary intake of flavones, flavonols, and flavanones in the USA was estimated by Kühnau [2] in 1976 to be ≈1 g/day, expressed as glycosides, which corresponds to about 170 mg of aglycones [10]. These values are probably too high, however, since they are based on analytical techniques now considered inadequate. Furthermore the flavonoid content of non-edible parts was sometimes included [5]. Recently, Hollman et al. [5] calculated the intake of flavonols and flavones in The Netherlands to be 23 mg/day (as aglycones). In the event, any comparisons between results and the techniques applied are not strictly valid unless the same sample is analyzed. For accurate work, the various components

Table 2
Concentrations of flavonols and isoflavones of selected foods (mg/100 g)

Food item	Flavonol, quercetin	Isoflavones[a]	Ref.
Onion	28–49	–	[3]
Apple	2.1–7.2	–	[3]
Lingonberry	7.7–14.6	–	[4]
Red wine	0.4–1.6	–	[59]
Soy bean	–	160	[6,33]
Tofu	–	30	[6]
Mung bean sprout	–	2.6	[4]

[a] Mainly genistein and daidzein.

need to be separated from the mixture and individually quantified.

We survey here the analytical techniques that are used for the quantitation of flavonoids and isoflavonoids based on the use of internal standards. We also review the synthesis, determination of isotopic purity, and use in quantitation of these polydeuterated compounds, conveniently prepared by direct D–H exchanges within the full flavonoid or isoflavonoid structure.

2. Methods for flavonoid and isoflavonoid quantitation

The product or extract to be examined often contains several different types of flavonoids or isoflavonoids which occur in foods as complex mixtures of aglycones, glycosides, and glycoside esters. In physiological samples they are present mostly as sulfate esters and glucuronide derivatives. This variation makes the quantitation difficult and, thus, in many isolation procedures, the conjugates are hydrolyzed before quantitation, which limits the number of analytes to a few aglycones.

The most common techniques for the determination of flavonoid contents are HPLC and GC with various detection systems. Usually methods such as LC–UV having a high detection limit have been used for matrices such as soybeans and supplemented plasma samples that contain high levels of the analyte. More sensitive methods are required if flavonoids or isoflavonoids are to be quantified in biological fluids or food products with only low contents of analytes. Table 3 shows the most commonly used methods of flavonoid or isoflavonoid quantitation with their sensitivity limits.

Since analytical work in flavonoid and isoflavonoid chemistry before 1970 was mainly focused on the separation and identification of compounds, most of the earlier quantitative data are inaccurate for methodical reasons. On average, the values published before 1970 are too high [2]. Herrmann and coworkers [11,12] quantified flavonoids spectrophotometrically in thin layer chromatography spots. The extraction and hydrolysis procedures were not optimized and the flavonoids were quantified as a group giving the sum of flavanones, flavones, and flavonols, for example. Crude quantitation of single components or mixtures can be done by UV–Vis spectrophotometry. The concentration is calculated from the Beer–Lambert law ($A = \varepsilon cl$), where ε is obtained using a standard solution or taken from the literature [13].

2.1. HPLC

Merken and Beecher [14] have reviewed the HPLC and sample preparation methods employed to quantify flavonoids and isoflavonoids, as reported in the literature from 1989 to early 1999. When these compounds are present at high levels, as in soy beans, conventional HPLC–UV methods are sufficient for quantitation. Xu et al. [15] have used HPLC to quantify isoflavonoids even in plasma after soymilk consumption. However, the detection limit (see Table 3) of this method is too high for the measurement of isoflavonoids in non-supplemented samples or in food products not containing large amounts of isoflavonoids. Diode array and coulometric detectors [16,17] have been used to increase the sensitivity. However, equol **14**, *O*-demethylangolensin **13,** and coumestrol **7**, which are present in low amounts in plasma, could not be detected with a coulometric detector [16]. Nurmi and Adlercreutz [18] quantified phytoestrogens in non-supplemented plasma samples using HPLC with coulometric electrode array detection. Recently, MS has also been used as a detection system with HPLC [19,20]. Cimino et al. [21] determined isoflavone aglycones and their sulfate and glucuronide derivatives in urine by LC–MS. Most HPLC studies have relied on external standards [22]; internal standards, where employed, were structurally related compounds such as flavone [23] or biochanin A [21] used in the quantitation of isoflavonoids. Holder et al. [24] employed deuterium-labeled internal standards in quantifying genistein **3** and daidzein **1** in rat blood by LC–MS, and provided thereby improved reliability over previous LC–MS methods in both accuracy and precision. They used [6,8,3′,5′-D_4]-genistein **18** (Scheme 1) as a reference for genistein **3,** but the ring A labels may not be stable in the procedure. The stable [2′,3′,5′,6′-D_4]-genistein **32** synthesized as reported by us [25] would be a better alternative in the HPLC–MS method.

Table 3
Comparison of selected quantitation techniques

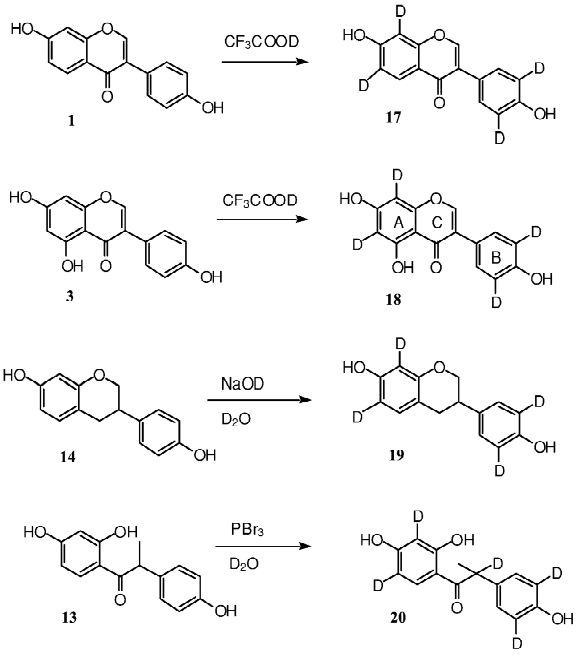
Matrix	Method	Flavonoids analyzed[a]	Internal standard	Sensitivity limit	Ref.
Human plasma	LC–UV	G, D	None	2 ng/20 ml, 390 n*M*[b]	[15]
Human urine	LC–diode array detector	G, D, E, O, F, B, C	Flavone	5–780 n*M*, 5 n*M*[b]	[23]
Human/rat urine	LC–MS	G, D, E, O, DHD, DHG, Gl, + conjug[c]	Biochanin A[d]	20 n*M*[b]	[21]
Rat blood	LC–MS–SIM	G, D	D-labeled[e]	≈5 n*M*	[24]
Human plasma	LC–coulometric electrode array detector	G, D, E, O, DHD, DHG, D7G, G7G	^{3}H-E_2-G[f]	2.5–9.3 n*M*, 4.6 n*M*[b]	[18]
Human plasma	ID-GC–MS–SIM[h]	G, D, E, O	^{3}H-E_2-G + D-labeled[e]	0.2–1.0 n*M*	[38]
Human plasma	TR-FIA[i]	G, D	^{3}H-E_2-G[f]	1.8 pg/20 ml 0.35 n*M*[b]	[31]
Vegetables and fruits	LC–UV	Q, K, M, L, A	None	1–4 mg/100 g	[22]
Soybean foods	LC–diode array detector	G, D, Gl + glug[g]	None	90 mg/100 g[b]	[6]
Legumes	LC–diode array detector	G, D, E, O, F, B, C	Flavone	6.5–34.5 mg/100 g 6.5 mg/100 g[b]	[23]
Various foods	ID-GC–MS–SIM	G, D, F, B, C	D-labeled[e]	2–3 mg/100 g	[9,33]

[a] G, genistein; D, daidzein; E, equol; O, *O*-demethylangolensin; F, formononetin; B, biochanin A; C, coumestrol; DHD, dihydrodaidzein; DHG, dihydrogenistein; D7D, daidzein-7-glucoside; D7G, genistein-7-glucoside; Q, quercetin; K, kaempherol; M, myricetin; L, luteolin; A, apigenin; Gl, glycitein.
[b] Value for daidzein.
[c] Glucuronides and sulfates.
[d] Added just before LC–MS.
[e] Deuterium-labeled internal standards for all analytes.
[f] Tritiated estradiol-17β-glucuronide, recovery calculated after hydrolysis and extraction.
[g] Glucosides, acetylglucosides and malonylglucosides for G, D and Gl; nine in total.
[h] Isotope dilution gas chromatography–mass spectrometry–selected ion monitoring.
[i] Time-resolved fluoroimmunoassay.

In general, derivatization is not required in HPLC methods and there are usually fewer purification steps than in GC–MS. Also, the hydrolysis of glycoside derivatives is not necessary, although in many HPLC studies this is done and only aglycones are measured.

2.2. Immunoassays

Immunoassays are specific protein binding assays which rely on the affinity of the recognition reaction between an antibody and the determinant in antigen. An antigen is formed by the attachment of a target analyte with a suitable functional group (e.g. genistein 4′-(carboxymethyl)ether) [26] to a macromolecular carrier protein. Rabbits or other animals immunized with an antigen form antibodies against it. Collected serum, labeled tracers, and samples (e.g. plasma) are then allowed to react and bind, and the free portions are washed away. For radioimmunoassay (RIA), a radiolabeled tracer (e.g.^{125}I-labeled) must be synthesized, and for fluoroimmunoassay (FIA), europium-labeled or other appropriate tracer. Radioactivity or fluorescence of the fractions is measured and quantitative results are obtained by comparing the counts against a standard curve. Analytes and labeled tracers compete for binding to the antibody. The higher the level of analyte in the sample the less tracer is bound. The use of immunoassays usually provides an increase in sensitivity (see



Scheme 1. Deuterium labeling of isoflavonoids.

Table 3) but decrease in specificity relative to GC–MS since cross-reactivity occurs, for example between genistein **3** and biochanin A **4** [27]. RIA has been used to quantify daidzein **1** [28] and genistein **3** [27] in human serum and formononetin **2** in wether's plasma, rumen fluid [29], murine plasma and mammary glandular tissue [30]. Immunoassays may be a good alternative in future for the quantitation of flavonoids and isoflavonoids, especially in serial work as in epidemiological studies and screenings, since they are fast and expedient and can be applied for very low levels of analyte (e.g. 1.8 pg detected in 20 ml sample) [31].

2.3. GC

GC has been used when there are large amounts of compounds in the matrix to be quantified. For example, lignans were measured in urine with cholestane as the internal standard [32]. GC is also still used in the study of food products containing very large amounts of lignans [33]. However, Adlercreutz and co-workers [34,35] found its sensitivity insufficient for urine samples containing low amounts of isoflavonoids.

2.4. ID-GC–MS–SIM

The use of gas chromatography–mass spectrometry (GC–MS) with stable isotope analogues as internal standards for the quantitation of compounds in biological matrices and food has increased dramatically. The literature relevant to the technique, published between 1970 and 1980, has been reviewed and critically evaluated by Garland and Powell [36].

In isotope dilution gas chromatography–mass spectrometry (ID-GC–MS), an accurately weighed amount of internal standard (I.S.) is added to the sample to be measured (e.g. food or biological fluid) before sample preparation. The analytes and I.S. are extracted, purified, derivatized, separated by gas chromatography (GC), and finally analyzed by mass spectrometry (MS). A major step forward in the use of MS in quantitative applications was the development of the selected ion monitoring (SIM) technique, which relies on the selective monitoring of just one or a restricted number of relevant m/z values. Compounds that do not generate ions at those specific values are not detected. The quantitation is achieved by comparing the peak area ratios of the analyte ions and deuterated I.S. ions, with the peak area ratios of the calibrators (corresponding to isoflavonoids and their D-labeled analogs) used in preparing a standard curve [35,33,37]. The entire procedure is laborious but the power of the technique is its sensitivity coupled with high specificity.

In the technique developed by Adlercreutz and co-workers [34,35], many lignans and isoflavonoids are simultaneously isolated, purified, and quantified by the isotope dilution GC–MS method in human urine [34,35], plasma [38], and feces [39]. In the procedures for urine and plasma, losses during the initial purification steps are compensated for using radioactive estrogen conjugates, and deuterium-labeled internal standards are added later. The D labels in deuterated genistein **18** used in these early studies were not stable enough and quantitation was done using the sum of M-15, M-16, and M-17 peaks in MS. Although the method produced acceptable

results, the need for a stable polydeuterated derivative was clear [34,35,38].

In the procedure for food products, the deuterium-labeled isoflavonoids are added to the samples at the beginning of the sample processing. This means that all the deuterium labels should be stable in the reference compounds. Stable polydeuterated isoflavonoids have been reliably used to quantify the isoflavonoids in various food products by ID-GC–MS–SIM [33,40,41].

3. Requirements for the internal standards (I.S.)

An internal standard is used to compensate for any losses during sample isolation, purification, derivatization, and separation, as well as of variations in the measurement process. A stable isotope labeled analogue of the analyte having the same retention time in GC but a different m/z value in MS is an optimal standard. Since the physicochemical properties of an isotopically labeled analogue are virtually identical with those of the analyte, no separation takes place during the procedure. The stable isotopes most commonly used in ID-GC–MS–SIM are D, ^{13}C, and ^{15}N. The most popular of the three is deuterium. Other possibilities for an internal standard are a close homologues of the analyte or a compound of the same chemical class [37,42]. Clearly, in the last two cases it has to be ensured that the chosen compound is not present in the analyte mixture and that its peak will not overlap with those of analytes [13].

Most of the analytes and standards to be investigated by ID-GC–MS–SIM technique are usually TMS-derivatized. TMS-derivatized polyhydroxy aromatics show fairly intense m+1, m+2, and m+3 ions in their mass spectrum owing to the large number of carbon and silicon atoms in the molecule. Thus, the molecular mass of the I.S. should preferably be at least three mass units higher to avoid interference by natural isotopes of the analyte at the m/z value of the labeled compound. A difference of more than four or five mass units may, however, cause chromatographic separation between I.S. and the analyte [37]. Clearly, no unlabeled species can be present and the deuterium labels must be stable under the analytical conditions employed [33,37]. Derivatization with labeled reagents such as $(CD_3)_3SiCl$ is not satisfactory since this derivatization is typically done only just before GC and losses before the derivatization step will not be accounted for [37]. Finally, the deuterium-labeled standard should be chemically, isomerically, and isotopically pure.

To sum up, HPLC and GC methods are comparable to the GC–MS methods for the analysis of flavonoids and isoflavonoids in food products with high concentration of analytes. However, with lower concentrations, in food or biological fluids, the more complicated ID-GC–MS–SIM method offers a greater sensitivity and specificity. The major advantage of LC over GC–MS methods is that there is no need for derivatization and only minimal pretreatment of sample is required. Although many HPLC methods have been described for the separation and quantitation of flavonoids and isoflavonoids in food extracts and biological fluids, few of them are sensitive enough for the measurement of all biologically interesting compounds. Not all investigators have used internal standards to adjust for the analyte loss in extraction and analysis. To ensure food quality assurance, internal standards, preferably D-labeled, should be used for all the compounds to be measured.

4. Deuteration

4.1. Isoflavonoids

Deuteration methods (Scheme 1) exchange aromatic protons that are *ortho* or *para* to the phenolic hydroxyl group. It was found that [6,8,3′,5′-D_4]-genistein **18** may rather easily lose the deuterium atoms in the highly activated positions in the ring A [35,43]. For the ID-GC–MS–SIM technique it is desirable that the D-labels are stable enough to withstand the isolation and purification procedures. In our work, a perdeuteration method was developed to produce [6,8,2′,3′,5′,6′-D_6]-genistein, in which also the less activated protons are exchanged for deuterons; a selective dedeuteration method was then applied to remove the labile deuteriums to obtain stable [2′,3′,5′,6′-D_4]-genistein **32** [44].

[8,3′,5′-D_3]-Daidzein 7-*O*-β-glucoside **21** [45], [8,3′,5′-D_3]-daidzein 4′-*O*-β-glucoside **22,** and

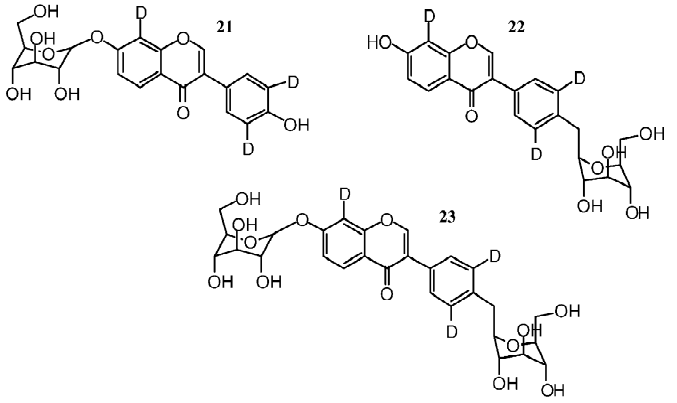


Fig. 1. Deuterated isoflavone glycosides.

[8,3′,5′-D_3]-daidzein 7,4′-di-*O*-β-glucoside **23** [46] (Fig. 1) were prepared from D_3-daidzein **35** [25], obtained using our $D_3PO_4 \cdot BF_3/D_2O$ deuteration reagent.

We have synthesized nine new stable deuterium-labeled isoflavonoids in good yield and isotopic purity with the $D_3PO_4 \cdot BF_3/D_2O$ deuteration reagent [25,44,47].

4.2. Flavonoids

H–D exchange of aromatic protons in the flavone, flavan, flavanol, and flavonol systems has been reported in a few papers. [6,8-D_2]-Apigenin **24** (Fig. 2) was prepared in weakly alkaline D_2O solution by Hand and Horowitz [48].

Kolar [49] reported the synthesis of [6,8-D_2]-catechin-5,7,3′,4′-tetramethyl ether ([6,8-D_2]-5,7,3′,4′-tetramethoxyflavan-3-ol) **25** and [6,8-D_2]-5,7,3′,4′-tetramethoxy-flavan **26** with D_2O–dioxan at 95 °C in Pyrex glass ampoules in the absence of any catalyst. Unexpectedly, the six and eight protons in 3-hydroxy-5,7,3′,4′-tetramethoxy-flavanone **27** did not exchange under the same conditions [49] (Fig. 3).

In the compounds mentioned above, only two protons were exchanged and the H–D exchange occurred at highly activated sites *ortho* to two OH or OMe groups. No exchange occurred at the aromatic ring containing only one OH group or two adjacent OMe groups. Furthermore, the deuteriums are probably not very stable in compounds **24–26** and compounds such as these could not be used as references, e.g. in GC–MS quantitation studies.

Little earlier work exists on the controlled synthesis of polydeuterated polyhydroxyflavones, and the only report involving an exchange within the intact target flavone deals with the deuterium labeling of rutin **28**, a 3-flavonol glycoside [50–52]. [6,8,2′,5′,6′-D_5]-Rutin **29** was obtained by treating rutin **28** twice with NaOH in D_2O at 95 °C for 8 h each time. However, the incorporation of D atoms at C-2′ and C-6′ was only 70%. Furthermore, owing to

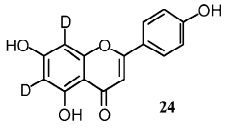


Fig. 2. [6,8-D_2]-Apigenin **24**.

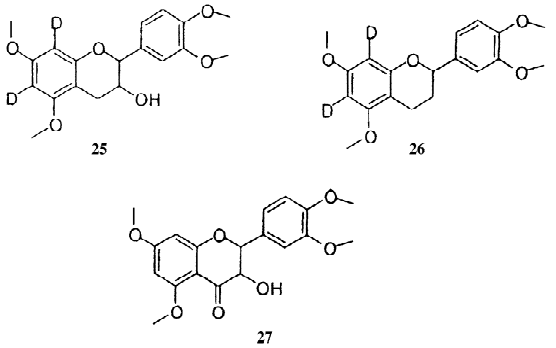
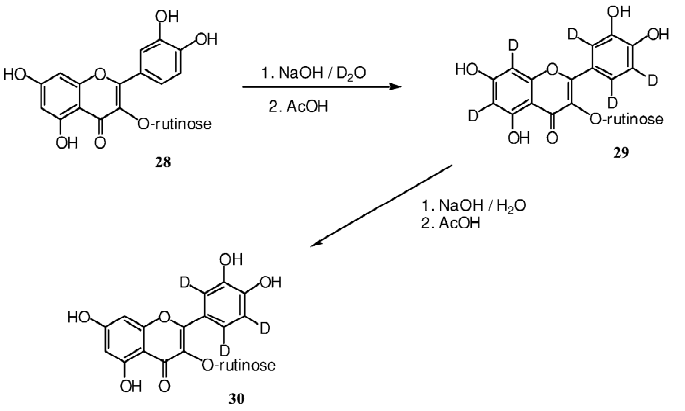


Fig. 3. Deuterated flavonoids **25**, **26** reported by Kolar. **27** failed to react [49].



Scheme 2. Deuterium labeling of rubin [50–52].

the lability of the 6- and 8-D substituents these were back-exchanged using NaOH in H_2O at 25 °C to give [2′,5′,6′-D_3]-rutin **30** in 60% yield (Scheme 2).

According to ^{1}H NMR, the D_1:D_2:D_3 ratio in the final product was 1:6:3 [50–52], which is not satisfactory for accurate ID-GC–MS–SIM work. Furthermore, just three D labels is probably not sufficient for the quantitation of a compound that contains five hydroxyl groups. The labeled rutin was nevertheless used for drug metabolism studies [51,52].

Our deuteration method, employing $D_3PO_4 \cdot BF_3$/D_2O, appears more effective and the dedeuteration method more selective for the preparation of stable, isotopically pure flavones and flavonols [53] than the one used in the deuteration of rutin [50–52].

5. Determination of isotopic purities

In the literature and in the catalogs of chemical suppliers the isotopic purity of a labeled compound is often unclear. Typically it is not specified how the determinations or calculations were made, or there may be contradictions in what the isotopic purity actually means. In some cases the percentage value of isotopic purity actually corresponds to the total isotopic content, not the percentage of an individual isotopomer. Most determinations have been done by MS, but there are no common rules on how to do the calculations. Millard [54] has discussed the calculation of the isotopic enrichment from MS data for compounds having a single atom of the heavy isotope. Biemann [55] has described a method for the calculation of isotopic composition for compounds having multiple labels of one mass unit heavier isotopes, but this approach is suitable only for compounds not having M-1 and M-2 fragments. De Leenheer et al. [36] have used a computer algorithm to calculate the contribution of each variant to the ion cluster of the labeled compound.

Flavonoids and isoflavonoids give intense molecular ion peaks and fragmentation via retro Diels–Alder process in MS. We calculate the isotopic purity (IP) of deuterated flavonoids and isoflavonoids from ion clusters in the molecular ion region in MS by comparison with those of undeuterated compounds as follows:

$$\mathrm{IP}\,\% = \frac{I_{m^+L}}{I_{m^+L} + (I_{(m-1)\,L} - I_{(m-1)\,U} - I_{(m-2)\,L} \times n) + I_{(m-2)\,L} + (I_{(m+1)\,L} - I_{(m+1)\,U})} \times 100$$

In the equation, I denotes the percentage intensity for the m^+ and for ions $m-1$, $m-2$ and $m+1$. Subscript L means labeled and U unlabeled compound, and n is the natural abundance of ^{13}C. The intensities of m^+ for both labeled and unlabeled compound were set to 100%. Since TMS-derivatized isoflavones usually show M-15 ($M–CH_3$) as the base peak, and as M-16 is very weak and no M-17 is present, isotopic purities are easily and reliably calculated from this region.

The analogous determination of isotopic purities

for certain deuterium labeled flavones and flavonols did not give results in accord with the NMR data. Possibly deuterium scrambling between two molecules can take place in EI–MS before the ionization [55]. We solved the problem by using negative ion electrospray (ES^-) LC–MS, which is a much more sensitive method and gave results in good accord with the NMR data. In LC–MS(ES^-) there were no M-1 or M-2 ions in the spectrum of undeuterated compound and the calculation of isotopic purities was easy. Guidugli et al. [56] have proposed that flavones have relatively intense $[M-1]^+$ fragments in EI–MS induced by the loss of hydrogen from ring B. They suggest a dibenzo-β-tropolone **31** ion as responsible for the fragment (Scheme 3) [56,57]. Such high intensity $[M-1]^+$ ions were however absent in our EI–MS spectra of undeuterated flavones, indicating that our problems with the M-1 and M-2 etc. ions in some deuterium labeled flavones are not explained in terms of this fragmentation.

5.1. NMR spectroscopy

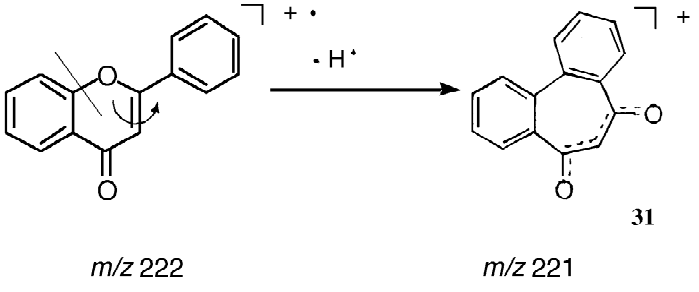
The sites of deuteration were determined from ^1H and ^{13}C NMR spectra by comparison with spectra of undeuterated compounds. Deuterium-carrying carbon atoms appear as low intensity triplets in the proton noise decoupled spectra and the carbon atoms of undeuterated compounds as intensive singlets (see Fig. 4). When signal assignments were ambiguous by chemical shift correlation alone, or when there were differences in assignment of protons and carbons of undeuterated compounds in the literature, assignments were based on 200 and 300 MHz HETCOR and GHMBC NMR data. HETCOR (heteronuclear shift correlation) and GHMBC (gradient selected heteronuclear multiple bond correlation) are both two-dimensional heteronuclear $^1H/^{13}C$ chemical shift correlations of which the first gives correlation over one bond and the second over two or three bonds.

6. Deuterated flavonoids and isoflavonoids

We have developed a method of perdeuteration with $D_3PO_4 \cdot BF_3/D_2O$ and removal of the labile labels with CH_3COCl–MeOH that proved highly suitable for isoflavonoids, flavones, and flavonols giving stable, isotopically and chemically pure deuterium labeled analogues in high yield [25,44,53,58]. With the perdeuteration–dedeuteration procedure, we obtained stable deuterated isoflavonoids [2′,3′,5′,6′-D_4]-genistein **32**, [2′,3′,5′,6′-D_4]-biochanin A **33**, and [3,2′,3′,5′,6′-D_5]-dihydrogenistein **34** (Fig. 5).

In addition, deuterated isoflavonoids [8,3′,5′-D_3]-daidzein **35**, [6,8,2′,3′,5′,6′-D_6]-daidzein **36**, [8,3′,5′-D_3]-formononetin **37**, [6,8,3′,5′-D_4]-dihydrodaidzein **38**, [3,6,8,3′,5′-D_5]-dihydrodaidzein **39**, and [2,4,8,10-D_4]-coumestrol **40** (Fig. 5) were prepared from the corresponding isoflavonoid with $D_3PO_4 \cdot BF_3/D_2O$ in high yield and isotopic purity [25,58]. For instance, our method of exchanging aromatic protons of the target isoflavone produced [8,3′,5′-D_3]-formononetin **37** in 96% yield and 91% isotopic purity [25], a considerable improvement over the total synthesis of 3′-5′-D_2-formononetin by Al-Ani and Dewick [58] where the yield was 27%.

Eight new deuterium-labeled flavones and flavonols (Fig. 6), [3,6,8,2′,5′,6′-D_6]-luteolin **41**, [3,2′,5′,6′-D_4]-luteolin **44**, [6,8,2′,5′,6′-D_5]-quer-



Scheme 3. Proposed $[M-1]^+$ fragment **31** for flavone [56,57].

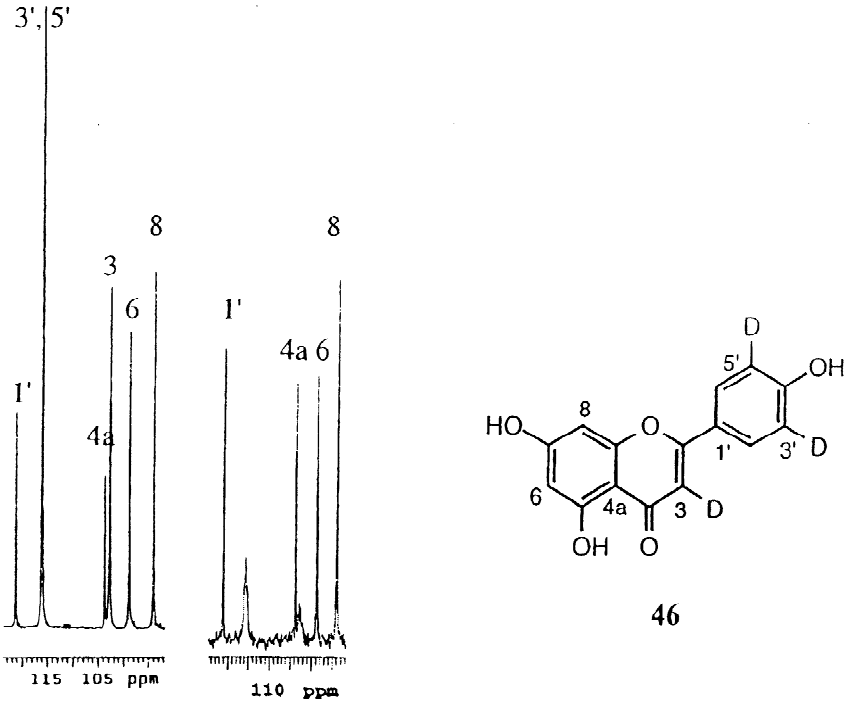


Fig. 4. Part of the ^{13}C NMR spectrum of apigenin **8** (left) and [3,3′,5′,-D_3]-apigenin **46** (right).

cetin **42**, [2′,5′,6′-D_3]-quercetin **45**, [3,6,8,3′,5′-D_5]-apigenin **43**, [3,3′,5′-D_3]-apigenin **46**, [8,2′,5′,6′-D_4]-fisetin **47**, and [4′,5′,6′-D_3]-2′,3′-dihydroxyflavone **48**, were also prepared by the $D_3PO_4 \cdot BF_3$/D_2O procedure and CH_3COCl–MeOH dedeuteration of the labile perdeutero derivatives (i.e. **41**→**44**; **42**→**45**; **43**→**46**) [53].

The reagent can be applied to other flavonoids and

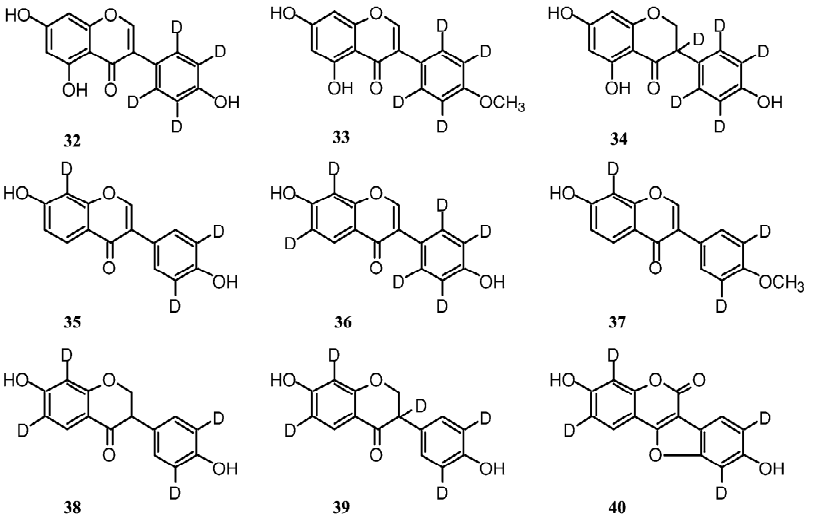


Fig. 5. New deuterium-labeled isoflavonoids [25,44,47].

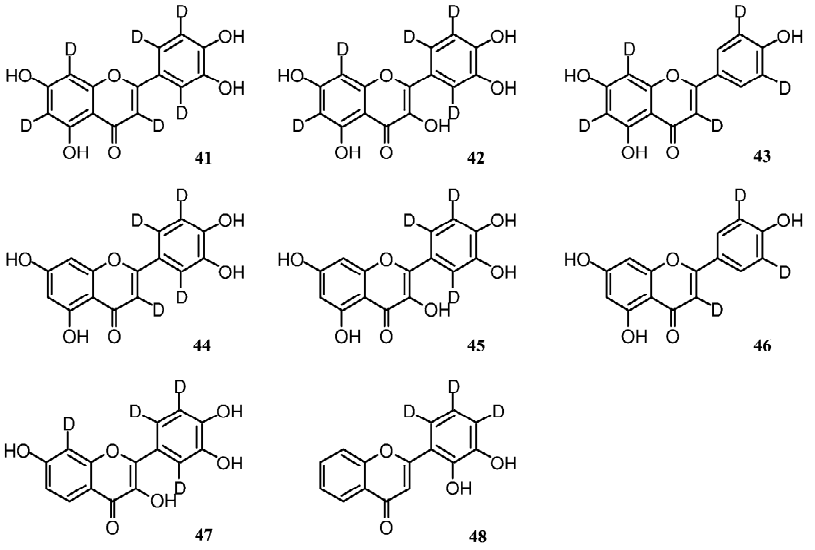


Fig. 6. New deuterium-labelled flavonoids [53].

presumably to many other types of aromatic structures. The new stable deuterium-labeled isoflavonoids have been successfully used in quantitation of the corresponding natural compounds in various foods [33,40,41]. The new compounds are also useful as internal standards in quantifying flavonoids in biological fluids and in metabolic studies.

7. Conclusion

Polydeuterated derivatives offer by far the best choice of reference compounds for the quantitation of flavonoids and isoflavonoids relying on isotopic labeling. In terms of reagent cost, number of synthetic steps required and number of heavier isotopes introduced, C-13 labeling is much less expedient. If judicious deuteration techniques are used, several D atoms can be exchanged for hydrogens in such a way that the resulting polydeutero derivatives are stable and do not lose any of the labels.

References

[1] S. Rasku, PhD thesis, University of Helsinki, 2000; K. Parikka, MSc thesis, University of Helsinki, 2001.
[2] J. Kühnau, World Rev. Nutr. Diet. 24 (1976) 117.
[3] M.G.L. Hertog, M.B. Katan, in: C.A. Rice-Evans, L. Packer (Eds.), Flavonoids in Health and Disease, Marcel Dekker, New York, 1998, p. 447.
[4] S.H. Häkkinen, S.O. Kärenlampi, I.M. Heinonen, H.M. Mykkänen, A.R. Törrönen, J. Agric. Food Chem. 47 (1999) 2274.
[5] P.C.H. Hollman, M.G.L. Hertog, M.B. Katan, Food Chem. 57 (1996) 43.
[6] H.-J. Wang, P.A. Murphy, J. Agric. Food. Chem. 42 (1994) 1666.
[7] K. Reinli, G. Block, Nutr. Cancer 26 (1996) 123.
[8] K.R. Price, G.R. Fenwick, Food Addit. Contam. 2 (1985) 73.
[9] W. Mazur, Bailliére's Clin. Endocrinol. Metab. 12 (1998) 729.
[10] S. Heinonen, K. Wähälä, H. Adlercreutz, Anal. Biochem. 274 (1999) 211.
[11] K. Herrmann, J. Food Technol. 11 (1976) 433.
[12] O.R. Gottlieb, in: J.B. Harborne, T.J. Marby, H. Marby (Eds.), Flavonoids, Chapman and Hall, London, 1975, p. 296.
[13] K.R. Markham, S.J. Bloor, in: C.A. Rice-Evans, L. Packer (Eds.), Flavonoids in Health and Disease, Marcel Dekker, New York, 1998, p. 1.
[14] H.M. Merken, G.R. Beecher, J. Agric. Food Chem. 48 (2000) 577.
[15] X. Xu, H.-J. Wang, P.A. Murphy, L. Cook, S. Hendrich, J. Nutr. 124 (1994) 825.
[16] A.A. Franke, L.J. Custer, W. Wang, C.Y. Shi, Proc. Soc. Exp. Biol. Med. 217 (1998) 263.
[17] K.D.R. Setchell, M.B. Welsh, J. Chromatog. 386 (1987) 315.
[18] T. Nurmi, H. Adlercreutz, Anal. Biochem. 274 (1999) 110.
[19] L. Coward, M. Kirk, N. Albin, S. Barnes, Clin. Chim. Acta 247 (1996) 121.

[20] S. Barnes, L. Coward, M. Kirk, J. Sfakianos, Proc. Soc. Exp. Biol. Med. 217 (1998) 254.
[21] C.O. Cimino, S.R. Shelnutt, M.J.J. Ronis, T.M. Badger, Clin. Chim. Acta 287 (1999) 69.
[22] M.G.L. Hertog, P.C.H. Hollman, D.P. Venema, J. Agric. Food Chem. 40 (1992) 1591.
[23] A. Franke, L.J. Custer, C.M. Cerna, K. Narala, Proc. Soc. Exp. Biol. Med. 208 (1995) 18.
[24] C.L. Holder, M.I. Churchwell, D.R. Doerge, J. Agric. Food Chem. 47 (1999) 3764.
[25] S. Rasku, J. Koskimies, T. Hase, K. Wähälä, Tetrahedron 55 (1999) 3445.
[26] N. Al-Maharik, S.A. Kaltia, K. Wähälä, Molecules Online 3 (1999) 20.
[27] O. Lapcik, R. Hampl, M. Hill, K. Wähälä, N. Al-Maharik, H. Adlercreutz, J. Steroid Biochem. Mol. Biol. 64 (1998) 261.
[28] O. Lapcik, R. Hampl, N. Al-Maharik, A. Salakka, K. Wähälä, H. Adlercreutz, Steroids 62 (1997) 315.
[29] W. Wang, Y. Tanaka, Z. Han, J. Cheng, J. Agric. Food Chem. 42 (1994) 1584.
[30] W. Wang, Proc. Soc. Exp. Biol. Med. 217 (1998) 281.
[31] G.J. Wang, O. Lapcik, R. Hampl, M. Uehara, N. Al-Maharik, H. Stumpf, H. Mikola, K. Wähälä, H. Adlercreutz, Steroids 65 (2000) 339.
[32] T. Fotsis, R. Heikkinen, H. Adlercreutz, M. Axelson, K.D.R. Setchell, Clin. Chim. Acta 121 (1982) 361.
[33] W. Mazur, T. Fotsis, K. Wähälä, S. Ojala, A. Makkonen, H. Adlercreutz, Anal. Biochem. 233 (1996) 169.
[34] H. Adlercreutz, T. Fotsis, C. Bannwart, K. Wähälä, T. Mäkelä, G. Brunow, T. Hase, J. Steroid Biochem. 25 (1986) 791.
[35] H. Adlercreutz, T. Fotsis, C. Bannwart, K. Wähälä, G. Brunow, T. Hase, Clin. Chim. Acta 199 (1991) 263.
[36] W.A. Garland, M.L. Powell, J. Chromatogr. Sci. 19 (1981) 392.
[37] A.P. De Leenheer, M.F. Lefevere, W.E. Lambert, E.S. Colinet, Adv. Clin. Chem. 24 (1985) 111.
[38] H. Adlercreutz, T. Fotsis, S. Watanabe, J. Lampe, K. Wähälä, T. Mäkelä, T. Hase, Cancer Detect. Prev. 18 (1994) 259.
[39] H. Adlercreutz, T. Fotsis, M.S. Kurzer, K. Wähälä, T. Mäkelä, T. Hase, Anal. Biochem. 225 (1995) 101.
[40] W.M. Mazur, J.A. Duke, K. Wähälä, S. Rasku, H. Adlercreutz, J. Nutr. Biochem. 9 (1998) 193.
[41] W. Mazur, K. Wähälä, S. Rasku, A. Salakka, T. Hase, H. Adlercreutz, Br. J. Nutr. 79 (1998) 37.
[42] W.F. Trager, J. Clin. Pharmacol. 26 (1986) 443.
[43] K. Wähälä, Polyphenols Actual. 16 (1997) 5.
[44] K. Wähälä, S. Rasku, Tetrahedron Lett. 38 (1997) 7287.
[45] P. Lewis, K. Wähälä, Molecules Online 2 (1998) 137.
[46] P. Lewis, Regioselective Synthesis of Isoflavone-*O*-Conjugates: *O*-Glucosides, *O*-Glucuronides and Fatty Acid Esters, PhD thesis, University of Helsinki, 1998.
[47] S. Rasku, K. Wähälä, J. Label. Comp. Radiopharm. 43 (2000) 849.
[48] E.S. Hand, R.M. Horowitz, J. Am. Chem. Soc. 86 (1964) 2084.
[49] G.F. Kolar, J. Label. Comp. Radiopharm. 7 (1971) 409.
[50] K. Hiraoka, T. Miyamoto, S. Baba, T. Furuta, J. Label. Comp. Radiopharm. 18 (1981) 613.
[51] S. Baba, T. Furuta, M. Horie, H. Nakagawa, J. Pharm. Sci. 70 (1981) 780.
[52] S. Baba, T. Furuta, M. Fujioka, T. Goromaru, J. Pharm. Sci. 72 (1983) 1155.
[53] S. Rasku, K. Wähälä, Tetrahedron 56 (2000) 913.
[54] B.J. Millard, Quantitative Mass Spectrometry, Heyden, London, 1978.
[55] K. Biemann, Mass Spectrometry: Organic Chemical Applications, McGraw Hill, New York, 1962, 204.
[56] F.H. Guidugli, M.J. Pestchanker, J. Kakva, P. Joseph-Nathan, Org. Mass Spectrom. 19 (1984) 502.
[57] F.H. Guidugli, C.E. Ardanaz, J. Kakva, M.E. Garibay, P. Joseph-Nathan, Org. Mass Spectrom. 21 (1986) 117.
[58] H.A.M. Al-Ani, P.M. Dewick, J. Chem. Soc. Perkin Trans. 1 (1984) 2831.
[59] M.G.L. Hertog, P.C.H. Hollman, B. van de Putte, J. Agric. Food Chem. 41 (1993) 1272.