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APPLICATION OF DFT B3LYP/GIAO AND B3LYP/CSGT METHODS FOR INTERPRETATION OF NMR SPECTRA OF FLAVONOIDS

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Accurate predictions of ^{1}H (rms.±0.1 ppm) and ^{13}C (rms.±2 ppm) NMR chemical shifts are achieved for several flavonoids belonging to flavanones, flavones and flavonols through empirical scaling of shieldings calculated from GIAO and CSGT theory by means of computationally inexpensive DFT B3LYP/6--31G**// B3LYP/6-31G** method. It was shown that the GIAO method better reproduces the experimental values of the chemical shifts for flavonoids studied than the CSGT method, especially for ^{1}H NMR signals. It is demonstrated on the basis of the method proposed that the experimental NMR data recorded during kaempferol oxidation can be assigned to the compound with 3(2H)-benzofuranone moiety. The results indicate that the calculated NMR chemical shifts can find application in rapid and unequivocal identification of unknown bioactive low-mass compounds in food.

INTRODUCTION

Currently, a high interest has been observed in natural antioxidants, especially in those from edible plants, which is evidenced by the escalating amount of published research works on natural antioxidants [Pellegrini *et al*., 2003; Dormann, 2003; Rassin *et al*., 2003; Sanchez-Muniz *et al*., 2003]. This interest is triggered by the knowledge that antioxidants play an important role in the food industry, increasing the shelf life of food products and improving the stability of lipids and lipid-containing foods. Natural antioxidants such as flavonoids play also a very important role in human health, preventing radical-induced diseases such as cancer, arteriosclerosis and other diseases [Sanchez-Muniz *et al*., 2003; Ren *et al*., 2003; Vessal *et al*., 2003; Prior, 2003; De Freudis *et al*., 2003; Bucki *et al*., 2003; Iwashina, 2003]. Flavonoids, usually with several phenolic OH groups, are present in plant kingdom in substantial amounts. In contrast to expectations, there is no simple relationship between antioxidant activity and the total number of phenolic OH groups or general molecular structure of flavonoids, at all. One of the factors influencing antioxidant free radical scavenging activity and biological activities are conformational preferences, intra- and intermolecular interactions as well as hydrogen bonds. Understanding of many phenomena related to the hydrogen bonds is still a challenge for both experimentalists and theoreticians. Application of theoretically computed NMR chemical shifts to organic structure determination have not yet become routine, despite the apparent capability to predict chemical shifts at sufficient level of accuracy for practical use [Cheeseman *et al*., 1996; Forsyth *et al*., 1996; Sebag *et al*., 2000].

When working with medicinal plants, fruits and vegetables, the main goal is to identify and isolate the biologically active compounds. In addition, it is also important from the toxicological point of view to determine the decomposition pathway of active compounds present in the medical plants [Jørgensen *et al*., 1998]. As an approach, a chemical screening using high performance liquid chromatography (HPLC) coupled with ultraviolet (UV) and mass spectroscopic (MS) detection at the earliest stage of separation can be performed [Wolfender *et al*., 1997]. However UV and MS information obtained on-line are limited with respect to the direct identification of complex metabolites within the crude extract [Wolfender *et al*., 1997]. For screening of unknown compounds, a technique complementary to LC/UV/MS is necessary. As nuclear magnetic resonance spectroscopy (NMR) is by far the most powerful tool for obtaining detailed structural information about compounds in solution, the coupling of LC with NMR detection seems to be the method of choice for on-line identification [Wolfender *et al*., 1997]. Furthermore, the wide combined use of LC/NMR together with quantum-chemical calculations is still waiting for future. The application of theoretically computed ^{1}H and ^{13}C chemical shifts to organic structure determination is now possible because the apparent capability for predicting shifts of both ^{1}H and ^{13}C as well as other nuclei at sufficient level of accuracy enables practical applications [Forsyth *et al*., 1997]. To achieve the goal of routine practical use the predicted ^{1}H and ^{13}C chemical shifts need to be accurate for a given molecule in a solution which includes a wide variety of functional groups and conformations. The prediction also needs to be achieved at the modest computational cost. Several methods used in frame of *ab initio* calculations are now available for calculating nuclear shieldings, such as the Gauge Including Atomic Orbitals (GIAO) [Cheeseman *et al*. 1996] and the Continuous Set of Gauge Transformations (CSGT) [Wolinski *et al*., 1990]. The Density Function Theory

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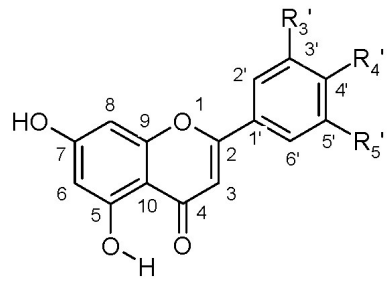
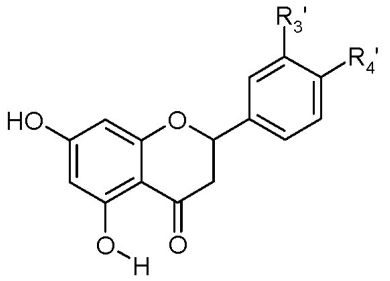
(DFT) method provides a low cost alternative for much more expensive *ab initio* methods.

In this study, we use the computational Gauge-Independent Atomic Orbital (GIAO) [Cheeseman *et al*., 1996] and the Continuous Set of Gauge Transformations (CSGT) [Wolinski *et al*., 1990] methods for calculating nuclear shieldings, for selected flavones (acacetin (R_3'=H, R_4'=OCH_3, R_5'=H), apigenin (R_3'=H, R_4'=OH, R_5'=H), chrysin (R_3'=H, R_4'=H, R_5'=H), and luteolin (R_3'=OH, R_4'=OH, R_5'=H)); flavanones (hesperetin (R_3'=OCH_3, R_4'=OH) and naringenin (R_3'=H, R_4'=OH), and flavonols (galangin (R_3'=H, R_4'=H, R_5'=H), kaempferol (R_3'=H, R_4'=OH, R_5'=H), myricetin (R_3'=OH, R_4'=OH, R_5'=OH) and quercetin (R_3'=OH, R_4'=OH, R_5'=H)) (Figure 1). As an example of the practical application of the theoretical computation we also report results of our computational study on oxidation pathway of kaempferol proposed by Jørgensen *et al*. [1998]. It will be demonstrated that the comparison of the results of our theoretical ^{1}H and ^{13}C NMR chemical shifts obtained for various possible oxidation products with experimental data enables more unequivocal identification of the products observed.



Flavones



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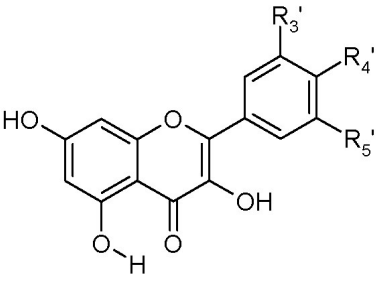


Flavonoles

FIGURE 1. Chemical structure and atom numbering systems of several groups of flavonoids studied.

METHODS

All geometry optimizations were performed using Gaussian 98 program package with the standard 6-31G** basis set using density functional theory (DFT) B3LYP method. In the case of oxidized kaempferol structures, the single-point energy calculations were performed with the 6-311G** basis on the optimized structures. In this work, theoretical values of the isotropic shieldings were obtained by means of computational GIAO//B3LYP/6-31G**//B3LYP/6-31G** as well as CSGT//B3LYP/6-31G**//B3LYP/6-31G** routes. The computed values of isotropic shieldings for various flavonoids were converted to theoretical values of chemical shifts by means of empirical scaling procedure [Forsyth *et al*., 1997] using a high resolution ^{13}C NMR spectra of quercetin taken as the reference. In this work, a linear regression found for quercetin was used to provide empirical scaling for theoretical isotropic shieldings in order to achieve more closely the level of accuracy needed for practical applications of computed ^{1}H and ^{13}C NMR data to different flavonoid derivatives and their isomers. The slope (s) and intercept (i) at the least-squares correlation line was used to scale GIAO or CSGT isotropic absolute shieldings (σ) to obtain predicted chemical shifts, δ_{pred} [Forsyth *et al*., 1997]

$$\delta_{pred} = s \cdot \sigma + i.$$

The theoretical values of the chemical shifts obtained were compared with the experimental ^{13}C NMR data for the compounds published mainly in recent papers [Wawer *et al*., 2001; Price *et al*., 1997, 1998]. The same procedure was also applied to the ^{1}H NMR data.

RESULTS AND DISCUSSION

Figure 2 is a typical example of the correlation between experimental ^{13}C NMR chemical shifts theoretical values of isotropic shieldings for quercetin computed by means of GIAO/B3LYP/6-31G** and CGS/B3LYP/6-31G** methods. It can be seen that in both cases theoretically derived quantities quantita-

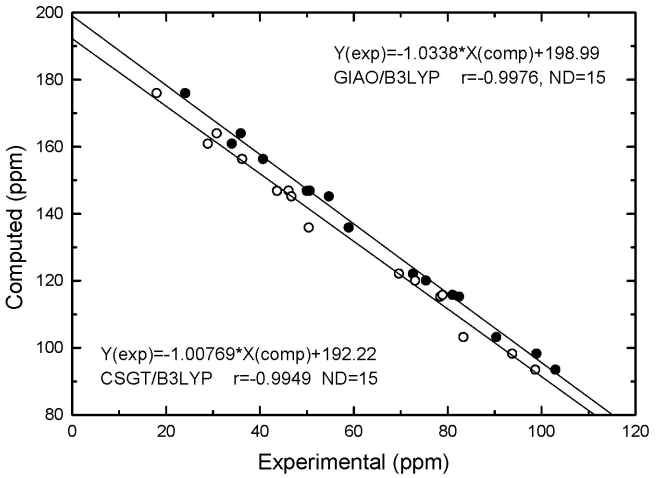


FIGURE 2. Correlation between experimental ^{13}C NMR chemical shifts and theoretical values of isotropic shielding for quercetin computed by means of GIAO/B3LYP/6-31G** (filled circles) and CSGT/B3LYP/6-31G** (open circles) method.

TABLE 1. Parameters of the linear least squares regression equation for correlation between experimental ^{13}C NMR chemical shifts [Wawer *et al*., 2001] and theoretical values of isotropic shieldings several flavonoids computed by means of GIAO/B3LYP/6-31G** and CSGT/B3LYP/6-31G** method.

Compound	GIAO/B3LYP/6-31G**		CSGT/B3LYP/6-31G**	
	Slope (s)	Intercept (i)	Slope (s)	Intercept (i)
Acacetin	-1.0311	198.89	-1.0172	193.56
Apigenin	-1.0407	199.36	-1.0350	194.42
Chrysin	-1.0350	198.50	-1.0284	193.94
Luteolin	-1.0515	200.03	-1.0435	194.49
Hesperetin	-1.0563	200.24	-1.0190	193.36
Naringenin	-1.0424	200.11	-1.0076	193.85
Galangin	-1.0265	199.16	-1.0023	193.34
Kaempferol	-1.0344	198.80	-0.9838	190.39
Myricetin	-1.0393	199.21	-1.0206	192.45
Quercetin	-1.0338	198.99	-1.0077	192.22

tively correlate with the experimental data allowing a proper assignment of the observed NMR signals. In each case, the experimental value of the ^{13}C chemical shift, δ(exp), linearly correlates with the computed isotropic shielding, σ(calc). The numerical values of the slope (s) and intercept (i) obtained by means of the least squares method for flavonoids studied are listed in Table 1. Detailed analysis of the numerical values of "s" and "i" allows us to state that the results of GIAO/B3LYP/6-31G** method are usually much better correlated with experimental data than those obtained by means of CSGT/B3LYP/6-31G** method.

TABLE 2. ^{13}C NMR chemical shifts of flavanones. Experimental data (in DMSO-d_6-D_2O) are from Wawer *et al*. [2001]. Computed values (in gas phase) were obtained using GIAO/B3LYP/6-31G** and CSGT/B3LYP/6-31G** method.

Carbon	Hesperetin			Naringenin		
	Exp.	GIAO	CSGT	Exp.	GIAO	CSGT
C-2	78.6	81.0	75.6	79.2	80.6	75.5
C-3	42.8	41.1	36.1	42.7	40.5	35.7
C-4	195.5	192.8	190.1	196.4	193.5	190.4
C-5	164.0	166.6	164.6	164.5	166.2	164.0
C-6	96.4	94.3	94.7	96.2	94.6	95.0
C-7	166.9	164.0	161.9	166.5	163.9	161.9
C-8	95.4	94.5	94.1	95.2	95.0	94.4
C-9	162.9	162.1	160.2	163.6	162.1	160.4
C-10	102.2	106.1	109.2	102.4	106.2	109.2
C-1'	131.2	132.7	127.9	130.0	130.4	127.2
C-2'	113.6	111.5	109.3	128.3	130.15	126.0
C-3'	146.6	147.2	147.9	115.4	113.4	112.0
C-4'	147.9	145.3	146.0	157.8	156.1	155.2
C-5'	111.5	109.0	107.6	115.4	113.8	110.8
C-6'	117.7	116.6	113.9	128.3	128.0	124.7
OCH_3	54.0	55.1	52.3			

The fits obtained by the least squares method between experimental and theoretical values of ^{13}C chemical shifts for hesperetin computed using GIAO/B3LYP/6-31G** and CSGT/B3LYP/6-31G** methods are as follows (r – correlation coefficient): GIAO, δ(exp) = 1.0086 δ(calc) - 0.572 (r=0.9987); CSGT, δ(exp) = 0.9942 δ(calc) + 2.919 (r=0.9971). It is seen in Table 2 that the values of δ obtained by means of GIAO procedure are more close to the experimental values than those that are derived *via* CSGT method. It conclusion seems to be general, therefore in the following part of this paper we present the numerical data obtained only *via* GIAO method.

In order to predict ^{13}C NMR chemical shifts of various flavonoids, we used empirical scaling procedure with quercetin as an arbitrary selected standard compound. The parameters of calibration equation for ^{13}C NMR spectra (s=-1.0338, i=198.99) are those given for Table 1 for quercetin. The comparison of the experimental values of ^{13}C NMR chemical shifts with those obtained theoretically for flavanones, flavones and flavonols are given in Tables 2-4, respectively. In each case, theoretical procedure with GIAO/B3LYP/6-31G** method can properly predict changes in the value of the chemical shift with variation in the structure of various flavonoids. It should be noted, however, that the presented method overestimates the predicted values of the ^{13}C chemical shift for C3 by *ca.* 2 ppm and for C10 even by *ca.* 4 ppm. On the other hand, it underestimates the predicted values of the ^{13}C chemical shift those for C6 and for C10 by *ca.* 2 ppm and even up to *ca.* 4 ppm, respectively. The comparison of experimental ^{13}C NMR spectra with those obtained theoretically for several flavonols given in Table 4 also confirm that the GIAO/B3LYP/6-31G** method can properly predict changes in the value of the chemical shift with variation in their structure.

Next, we have applied the same empirical scaling procedure to ^{1}H NMR data. In this case we have also selected quercetin as the standard. Figure 3 is a typical example of the correlations between experimental ^{1}H NMR chemical shifts theoretical values of isotropic shieldings chemical shifts for quercetin computed by means of GIAO/B3LYP/6-31G** method. It can be seen that in both cases theoretically derived quantities quan-

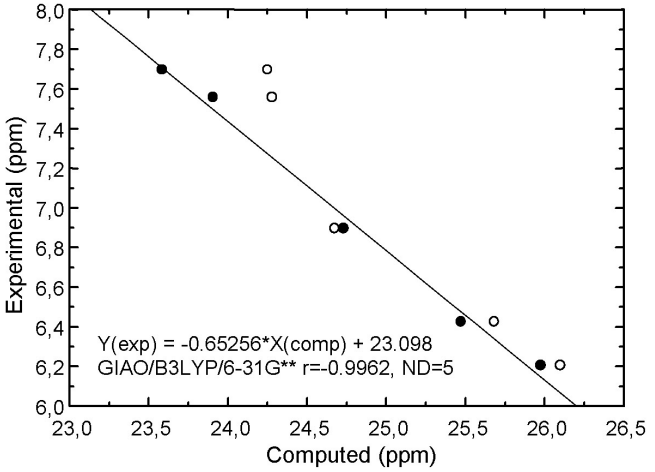


FIGURE 3. Correlation between experimental ^{1}H NMR chemical shifts and theoretical values of isotropic shieldings for quercetin computed by means of GIAO/B3LYP/6-31G** (filled circles) and CSGT/B3LYP/6-31G** (open circles) method.

TABLE 3. ^{13}C NMR chemical shifts of flavones. Experimental data (in DMSO-d_6-D_2O) are from Wawer *et al*. [2001]. Computed values (in gas phase) were obtained using GIAO/B3LYP/6-31G** method.

Carbon	Acacetin		Apigenin		Chrysin		Luteolin	
	Exp.	Comp	Exp.	Comp	Exp.	Comp.	Exp.	Comp
C-2	164.8	164.9	164.1	163.2	164.4	165.4	163.9	163.9
C-3	103.9	106.3	102.8	104.9	105.1	108.0	102.5	106.0
C-4	182.3	180.3	181.8	180.2	181.9	180.5	182.5	180.2
C-5	162.2	165.4	161.1	165.2	161.4	165.6	161.8	165.6
C-6	99.4	97.3	98.8	97.3	98.9	97.5	98.7	97.3
C-7	163.9	161.0	163.0	161.1	163.2	161.3	164.8	161.1
C-8	94.3	92.0	94.0	92.1	94.1	93.2	93.6	91.73
C-9	157.9	158.4	157.3	158.3	157.4	158.5	158.0	158.1
C-10	104.4	107.9	103.7	107.9	104.0	108.1	103.9	107.9
C-1’	123.5	125.6	121.3	124.5	130.7	133.7	122.3	124.3
C-2’	128.4	128.0	128.4	127.5	126.4	126.4	112.8	110.6
C-3’	114.8	118.1	116.0	113.5	129.1	128.4	145.6	142.9
C-4’	162.8	161.8	161.5	158.5	132.0	130.6	149.6	148.8
C-5’	114.8	109.4	116.0	115.5	129.1	128.5	115.4	115.9
C-6’	128.4	128.8	128.4	129.2	126.4	126.8	118.9	121.3
OCH_3	56.1	55.4						

TABLE 4. ^{13}C NMR chemical shifts of flavonols. Experimental data (in DMSO-d_6-D_2O) are from Wawer *et al*. [2001]. Computed values (in gas phase) were obtained using GIAO/B3LYP/6-31G** method.

Carbon	Galangin		Kaempferol		Myricetin		Quercetin	
	Exp.	Comp	Exp.	Comp.	Exp.	Comp.	Exp.	Comp.
C-2	146.5	146.5	146.8	146.8	146.9	146.6	146.9	146.7
C-3	138.0	139.7	135.7	138.6	135.9	138.7	135.9	138.0
C-4	177.1	174.6	175.9	174.2	175.8	174.1	176.0	174.1
C-5	161.6	163.9	160.7	163.7	160.8	163.7	160.9	163.8
C-6	99.2	96.8	98.2	96.9	98.2	96.8	98.3	96.7
C-7	165.1	162.2	163.9	161.9	163.9	162.0	164.0	161.9
C-8	94.4	92.6	93.5	92.8	93.3	92.9	93.5	92.6
C-9	157.3	157.1	156.2	157.1	156.1	157.1	156.3	156.9
C-10	104.1	105.7	103.1	105.4	103.0	105.6	103.2	105.6
C-1’	130.7	132.4	121.7	124.9	120.8	124.6	122.2	123.9
C-2’	128.4	129.8	129.5	131.8	107.2	106.4	115.3	113.8
C-3’	129.3	128.4	115.5	113.4	145.8	142.6	145.2	142.5
C-4’	131.8	128.8	159.2	157.3	135.9	133.8	146.8	147.3
C-5’	129.3	127.7	115.5	114.9	145.8	146.2	115.8	115.2
C-6’	128.4	125.4	129.5	128.3	107.2	107.5	120.1	121.1

titatively correlate with the experimental data allowing a proper assignment of the observed ^1H NMR signals.

It is seen in Figure 3 that the values obtained by means of GIAO procedure are more close to experimental values than those derived *via* CSGT method. This conclusion seems to be general. Therefore, in the following part of this paper, we present the numerical data obtained only *via* GIAO method. The parameters of calibration line for ^1H NMR spectra (s=-0.6526, i=23.098). The fit obtained by the least squares method between experimental and theoretical values of ^1H chemical shifts for kaempferol computed using GIAO/B3LYP/6-31G** method obtained using the above calibration line parameters is as follows: $\delta(exp) = 1.0086\ \delta(calc) - 0.572$ (r=0.9987).

Very little is known about the metabolism and potential toxic metabolites that could be produced from plant polyphenolics. In general, one of major difficulties is the lack of

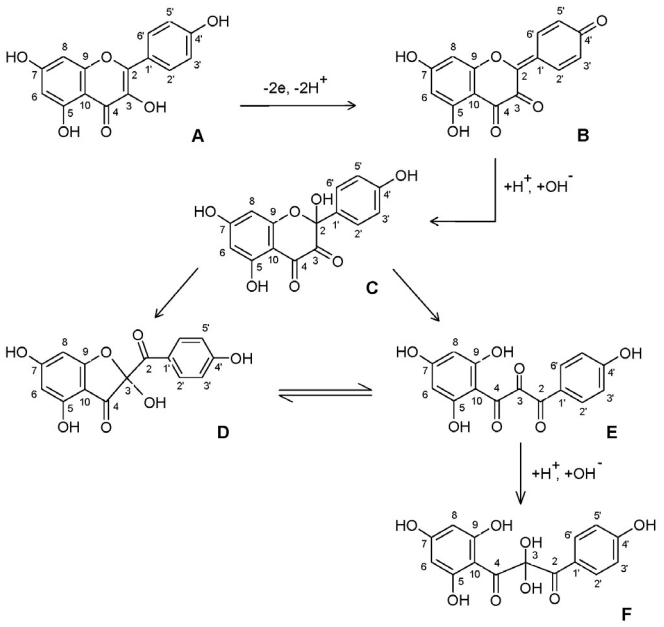
a reliable analytical method for quantifying quinone products formation from P450-catalysed oxidation processes [Bolton *et al*., 1998; Moridani *et al*., 2001]. Therefore, unequivocal identification of the oxidation products of often-consumed polyphenols is needed to form the basis for biomarkers for the fate of polyphenols in humans. As NMR spectroscopy is by far the most powerful tool for obtaining detailed structural information about compounds in solution, the coupling of LC with NMR detection seems to be the method of choice for on-line identification. A very good agreement between theoretical and experimental values for both ^{1}H and ^{13}C NMR chemical shift for flavonoids (shown in Tables 1-3 and Figures 2 and 3) prompted us to do the same with both ^{1}H and ^{13}C NMR data of the products obtained as a results of oxidation of flavonoids. Figure 3 is just an example of the application of the method for identification of the structure described as the oxidized

TABLE 5. ^{13}C NMR chemical shifts of kaempferol and some possible oxidized kaempferol molecules. Experimental data (in DMSO-d_6-D_2O) are from Jørgensen *et al*. [1998]. Computed values (in gas phase) were obtained using GIAO/B3LYP/6-31G** method. Numbering of carbon atoms is the same as that in Figure 1. Computed values are based on the calibration line determined for quercetin in Figure 2 and Table 1.

Carbon	Kaempferol		Oxidized kaempferol			
	Exp.	Comp.	Exp.	C	D	E
C-2	146.7	146.8	104.5	108.2	193.7	109.6
C-3	135.6	138.6	189.8	192.5	196.0	191.5
C-4	175.8	174.2	190.2	179.7	190.7	199.2
C-5	160.6	163.7	168.4	169.3	170.8	160.2
C-6	98.1	96.9	96.5	97.4	95.4	94.8
C-7	163.8	161.9	171.8	165.4	165.9	167.3
C-8	93.4	92.8	90.3	95.3	95.0	91.1
C-9	156.1	157.1	158.5	161.2	164.0	172..0
C-10	103.0	105.4	100.4	110.0	108.8	103.8
C-1’	121.6	124.9	124.9	129.1	126.7	127.4
C-2’	129.5	131.8	117.3	130.6	133.0	134.2
C-3’	115.4	113.4	144.7	112.6	116.0	114.8
C-4’	159.1	157.3	151.3	157.7	161.9	160.4
C-5’	115.4	114.9	114.8	114.5	112.7	111.9
C-6’	129.5	128.3	123.7	128.5	134.8	135.4

TABLE 6. Experimental and computed ^{1}H NMR chemical shifts for oxidized kaempferol. Experimental data (in DMSO-d_6-D_2O) are from Jørgensen *et al*. [1998]. Computed values (in gas phase) were obtained using GIAO/B3LYP/6-31G** method. Symbols are the same as those in Figure 4.

Structure	C-6	C-8	C-2’	C-3’	C-5’	C-6’
C	6.33	5.94	7.36	6.76	6.76	7.36
D	6.22	5.90	7.72	6.81	6.81	7.72
E	6.20	5.92	7.86	6.74	6.74	7.86
Exp.	5.96	5.91	8.03	6.84	6.84	8.03



Scheme 1. Simplified scheme of oxidation pathway for kaempferol (A), based on Jørgensen *et al*. [1998].

kaempferol. Both, experimental data for kaempferol and oxidized kaempferol (Tables 5 and 6) as well as the proposed oxidation pathway shown in Scheme 1 were reported by Jørgensen *et al*. [1998]. It is not clear which compound is observed in NMR experiments **C**, **D** or **E**.

It is seen in Tables 5 and 6 that comparison of experimental data with those calculated for **C** and **D** indicates no accurate agreement in any case. On the basis of B3LYP/6-311G** calculations one can state that species **E** (with 3(2H)-benzofuranone moiety) is more stable by *ca*. 8 kcal/mol than **C** while **E** is more stable than **D** by *ca*. 4.5 kcal/mol. Molecular modeling allows stating that rearrangement from **C** to isomers **D** and **E** can have place by simple proton transfer from C2-OH to O=C3 or to O1, respectively.

CONCLUSIONS

We have found that in all cases the theoretically derived ^{1}H and ^{13}C NMR chemical shifts linearly correlate with the experimental ones allowing for a proper assignment of the observed ^{1}H and ^{13}C NMR signals at relatively low computation cost. It was shown that the DFT B3LYP/GIAO method better reproduces the experimental values of the chemical shifts for flavonoids than the DFT B3LYP/CSGT method, especially for ^{1}H NMR signals. We have found that accurate predictions of both ^{1}H (rms.±0.1 ppm) and ^{13}C (rms.±2 ppm) NMR chemical shifts computed by DFT B3LYP/GIAO method can be achieved for various groups of flavonoids. In conclusion we can state that the accurate predictions of the location of NMR signals can be used as a valuable source of information on the chemical structure of flavonoids isolated from the natural sources as well as their transformation during storage or ingestion by living organisms. The results also indicate that the computed values of the chemical shifts could be practically on-line combined with experimental LC/NMR chemical shifts allowing for rapid and unequivocal identification of unknown flavonoids or other bioactive low-mass compounds in plants.

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