Available online at www.sciencedirect.com
ScienceDirect
Food Chemistry 107 (2008) 962–969
Food Chemistry
www.elsevier.com/locate/foodchem

Analytical Methods

Intermolecular interactions in phytochemical model systems studied by NMR diffusion measurements ☆

Stefano Tiziani [a], Steven J. Schwartz [b], Yael Vodovotz [b,*]

[a] CR UK Institute for Cancer Studies, University of Birmingham, Henry Wellcome Building for Biomolecular NMR Spectroscopy (HWB-NMR), Vincent Drive Edgbaston, Birmingham B15 2TT, United Kingdom

[b] Department of Food Science and Technology, The Ohio State University, Parker Food Science and Technology Building, 2015 Fyffe Road, Columbus, OH 43210, USA

Received 27 June 2007; received in revised form 22 July 2007; accepted 28 August 2007

Abstract

Novel functional foods, such as tomato juice with soy, represent a new strategy to increase consumption of health promoting ingredients and phytochemicals. Interactions between soy protein, isoflavones, and tomato carotenoids could impact the bioaccessibility and bioavailability of individual phytochemicals. The primary objective of this study was to assess possible interactions between daidzein and daidzin, soy protein and carotenoids using proton one-dimensional and two-dimensional pulsed field gradient nuclear magnetic resonance spectroscopy experiments.

The NMR results on phytochemical model systems indicate that the affinity between the daidzin and the soy protein isolate are higher as compared to the daidzein dissolved in the same model system. Two contributions to the interactions between the phytoestrogens and the protein were detected. The first involved intermolecular hydrogen bonding from hydroxyl groups. The second resulted in the shifting of the NMR signal related to the proton on the pyranone ring of isoflavones, suggesting a hydrophobic interaction. Diffusion NMR measurements showed that the addition of carotenoids to the soy isoflavones/protein mixture did not affect the diffusion coefficient of the polyphenols.

Keywords: NMR spectroscopy; Phytochemical; Isoflavones; Carotenoids; Diffusion measurements

1. Introduction

Phytochemicals are biologically active compounds present in fruit and vegetables that exhibit protective and disease-preventing properties (Johnson & Williamson, 2003). These compounds have recently garnered much attention due to epidemiological studies showing greater oxidative damage in population with diets deficient in fruits and vegetables (Giovannucci, 2002; Steinmetz & Potter, 1996). Novel functional foods, such as tomato juice with soy, represent a new strategy to achieve a higher consumption of health promoting ingredients such as soy protein, and phytochemicals such as isoflavones and carotenoids (Hasler, Bloch, Thomson, Enrione, & Manning, 2004; Rochfort, 2005; Tiziani & Vodovotz, 2005a, 2005b).

Lycopene is the predominant carotenoid in tomatoes, followed by minor compounds such as phytoene, β-carotene, and others (Khachk, Beecher, Goli, & Lusby, 1992; Tiziani, Schwartz, & Vodovotz, 2006). Several studies have shown lycopene (Fig. 1) to have a superior ability to quench singlet oxygen in comparison to all major carotenoids found in other fruits and vegetables (Clinton, 1998; Gann et al., 1999). The most abundant isoflavones found in soy beans are glycitein, genistein, and daidzein (Fig. 1), which are conjugated to malonyl-, acetyl- and

☆ This material is based upon work supported by USDA under Award No. 2001-52102-11333.
* Corresponding author. Tel.: +1 614 247 7696; fax: +1 614 292 0218.
E-mail address: vodovotz.1@osu.edu (Y. Vodovotz).

0308-8146/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2007.08.089

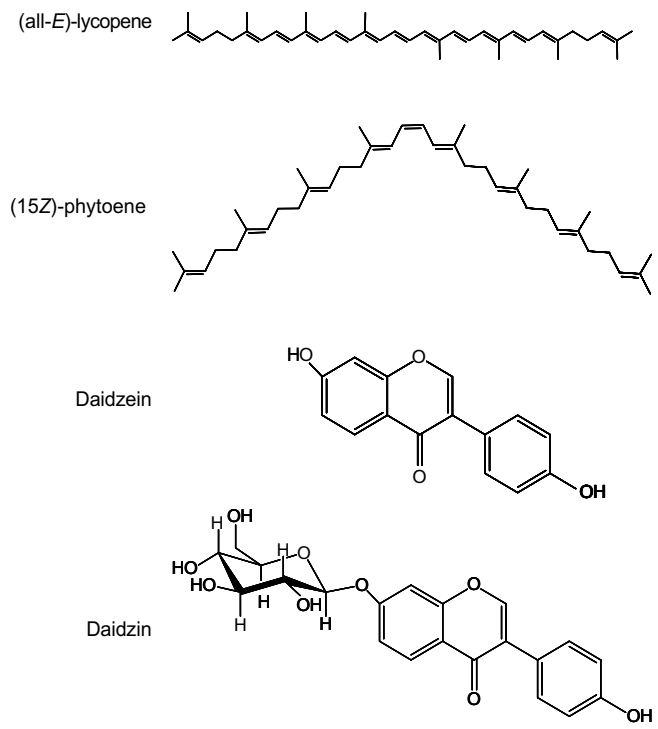

Fig. 1. Chemical structure of carotenoids (all-E)-lycopene and (15Z-phytoene) and isoflavones (daidzein and daidzin) present in the phytochemical model systems.

glucosides forms rather than in the aglycone ones (Dhaubhadel, McGarvey, Williams, & Gijzen, 2003; Tsangalis, Ashton, Stojanovska, Wilcox, & Shah, 2004). Sugar molecules can bind to various positions in the polyphenol rings, although there is a preference for the C3 positions via a β-glycosidic bond (Kroll, Rawel, & Rohn, 2003). The structural similarities of isoflavones, such as genistein and daidzein, to naturally occurring human estrogens suggest that these compounds may protect against hormone-dependent cancers (i.e., prostate and mammary) by suppressing the activity of estrogen. The relative proportions of isoflavone conjugates can vary considerably since malonyl and acetyl glycosides are susceptible to heat and can be readily hydrolyzed and converted to the more stable non-acetylated β-glycoside (Barnes, Kirk, & Coward, 1994; Cassidy, Hanley, & Lamuela-Raventos, 2000).

In 1999, the Food and Drug Administration (FDA) approved a health claim, petitioned by Protein Technologies International, for soy protein and its role in reducing levels of LDL and HDL/total cholesterol and thus the risk of coronary heart disease (FDA, 1999). Notably, increased consumption of soy protein has been linked to heart health and a claim to that effect has been issued by the FDA. Globulins glycinin (11S) and β-conglycinin (7S) are the major components of soy protein isolate (Arrese, Sorgentini, Wagner, & Anon, 1991). 11S has an estimated molecular weight (M_r) of 300,000 and consists of acidic polypeptide chains ($M_r \sim 37{,}000$–$40{,}000$) and basic polypeptide chains ($M_r \sim 19{,}900$–$20{,}000$) (Nielsen, 1985). 7S has a trimeric structure having a $M_r \sim 140{,}000$–$170{,}000$ and consist of subunits that varies form $M_r \sim 76{,}000$–$42{,}000$ (Brooks & Morr, 1985).

The impact of combining these potential health promoting active components into new products could affect absorption, metabolism, bioaccessibility and bioavailability of the individual phytochemical. There is little information about interactions of carotenoids and minor phenolic compounds with main constituents such as proteins, although they occur frequently in foods. Notable exceptions are a few studies in which the chemical reactivity and the types of interactions of phenolic and flavonoid compounds in mixture with proteins were investigated in model systems (Rawel, Czajka, Rohn, & Kroll, 2002; Rawel, Kroll, & Hohl, 2001). Phenolic compounds interact with amino groups such as lysine and tryptophan present in soy glycinin (Rawel et al., 2002). Additionally, previous findings indicated that protein–phenolic interactions are dependent on many factors such as amino acid composition and molecular size (Rawel et al., 2002). There are four potential types of interactions of phenolic compounds and protein: hydrogen, hydrophobic, ionic, and covalent bondings. The phenolic hydroxyl group is an excellent hydrogen donor and forms strong hydrogen bonds with the amide carbonyl of the peptide backbone (Siebert, 1999). Rawel et al. (2002) found that the reactivity of flavonoids in the presence of soy glycinin depends mainly on the position of phenolic hydroxyl groups and it increases with their number (Rohn, Rawel, Rober, & Kroll, 2005).

Recent studies have reported that interactions between polyphenols and proline-containing peptides may involve formation of π-bonded complexes in which the rings of two compounds overlap (Baxter, Lilley, Haslam, & Williamson, 1997; Bianco, Chiacchio, Rescifina, Romeo, & Uccella, 1997). Phenolic acids in comparison to the flavonoids were found to be more reactive towards free amino groups in soy glycinin. Ionic bondings were excluded in interactions between protein and polyphenols as demonstrated by Asano, Shinagawa, and Hashimoto (1982). In general, in polyphenolic compounds, other interactions such as hydrophobic bondings are more important for stabilization of the complexes formed, whereby proline residues seem to play a key role (Baxter et al., 1997; Siebert, 1999).

Pulsed-field-gradient spin-echo (PGSE) is a sensitive NMR technique especially for studying interactions of low molecular weight molecules, such as isoflavones and carotenoids, with high molecular weight macromolecules, such as protein (Gounarides, Chen, & Shapiro, 1999; Lucas & Larive, 2004). This technique combines the selectivity of high resolution NMR with pulse field gradients to enhance characterization on the basis of molecular diffusion coefficients (Johnson, 1999; Price, 1998). Many PGSE techniques have been developed to facilitate the identification of components in complex mixtures and assess possible interactions and/or aggregation phenomena (Jerschow & Muller, 1997; Morris & Johnson, 1992; Morris, Stilbs, &

Johnson, 1994). Translational diffusion is measured by application of an encoding followed by a decoding gradient. The encoding gradient serves to spatially label molecules which then diffuse through the solution for a specified time. A decoding gradient is applied to reverse the phase change of the encoding gradient. For those molecules that diffuse to different regions of the solution in the period between these two gradients, the decoding gradient will be unable to reverse the phase encoding (Johnson, 1999) resulting in the NMR signal decaying exponentially according to the self-diffusion behavior of individual molecules. Diffusion-ordered 2D NMR spectroscopy (DOSY) is a 2D-display mode of processed multicomponent PGSE experiments introduced by Morris and Johnson (1992). DOSY utilizes inverse Laplace transforms (Morris et al., 1994) to correlate the chemical shift values of specific components, displayed in one dimension (usually *x*-axis), with the diffusion coefficients displayed in the other dimension (usually *y*-axis) (Johnson, 1999). DOSY processing method is an attractive and versatile tool able to differentiate translational diffusion coefficients for fully resolved resonances (Morris & Johnson, 1992; Morris et al., 1994). This technique provides a unique means of establishing intermolecular interactions to study ligand–protein binding in several fields including analytical, environmental and pharmaceutical areas.

The primary objective of this study was to assess possible interactions between standard daidzein and daidzin, soy protein, and carotenoids extracts using PGSE experiments. These two isoflavones were chosen since daidzin was found to be the most abundant β-glucoside isoflavone, and daidzein the second most abundant aglycone isoflavone in tomato juice with soy germ. Several model systems prepared in dimethylformamide (due to its ability to dissolve a significant proportion of both the lipophilic and hydrophilic components) were used to assess possible interaction/competition between the two isoflavones, the soy protein and carotenoids extracted from tomato juice.

2. Materials and methods

2.1. Preparation of phytochemical model systems

The isoflavones used in this study were purchased from LC Laboratories (Woburn, MA), and include daidzein and daidzin (purity >99%). Soy protein isolate (PTI, St. Louis, MO) was denatured by retorting a saturated soy-protein-isolate aqueous solution at 100 °C for 15 min to reproduce the manufacturing conditions of the product (Tiziani & Vodovotz, 2005a, 2005b). No endothermic peak was visible in a DSC (DSC 2920 TA Instruments, New Castle, DE) analysis of the processed soy aqueous solution indicating that the protein was denatured (Puppo & Añón, 1999). The denatured soy protein was dialyzed (Spectra/Por molecular porous membrane MWCO: 8000, Spectrum Medical Industries Inc., Houston, TX) for two days under agitation to eliminate traces of salts, oligomers and other water soluble compounds. Solubility, hydrophobicity, and other chemical physical properties of soy protein isolate are reported in previous studies (Tiziani & Vodovotz, 2005a, 2005b). Finally, the purified soy protein was dried using a freeze drier; no trace of "freezable" water (endotherm ~0 °C) was detected analyzing the dried protein using DSC. Carotenoids from tomato juice were extracted according to the rapid procedure described in Tiziani et al. (2006) and dried under a steam of nitrogen at ambient temperature.

Several model systems were prepared dissolving the above cited compounds in dimethylformamide-d_7 (DMF, Sigma–Aldrich, Milwaukee, WI) in the following solutions:

Solution 1: 1 mg of daidzein or daidzin was dissolved in 1 ml of DMF-d_7;
Solution 2: *Solution 1* was saturated with denatured and dialyzed SPI with subsequent centrifugation for about 10 min. The supernatant solution was removed and used for analysis (the soy protein isolate solubility in DMF-d_7 was ~0.1 mg/ml).
Solution 3: ~0.2 mg/ml of dried tomato carotenoids were dissolved in *Solution 2*.
Solution 4: 1 mg of daidzin was dissolved in *Solution 1* containing 1 mg of daidzein.
Solution 5: ~0.1 mg/ml of SPI was dissolved in *Solution 4*.
Solution 6: ~0.2 mg/ml of dried tomato carotenoids were dissolved in *Solution 5*.

In addition to DMF, different solvents available in deuterated forms, such as acetone, hexane, methanol and DMSO, were evaluated for their ability to separately dissolve isoflavones, soy protein and carotenoids. DMF is a polar aprotic solvent; it is characterized by a high dielectric constant (36.7) and has the ability to dissolve lipid soluble compounds without acting as an oxidant; therefore it was considered an appropriate deuterated solvent to study interactions between phytochemicals and soy protein in model systems. Solutions 1–6 were used as model systems and not necessarily a reproduction of the protein/isoflavone and carotenoid concentrations found in the tomato juice with soy.

2.2. Nuclear magnetic resonance measurements

All NMR experiments were carried out using a Bruker 800 MHz (Bruker Biospin, Rheinstetten Germany) spectrometer equipped with a 5 mm TXI cryoprobe. The spectra were recorded at ambient temperature (293.15 K). Proton spectra were referenced to the TMS signal (δ 0.00 ppm). Chemical shift of phytochemicals were also compared with commercial standards daidzein, daidzin, and (all-E)-lycopene (from Sigma Chemical Co., St Louis, MO) dissolved in 0.6 ml of DMF-d_7.

All one-dimensional ^{1}H NMR experiments were measured applying the following identical conditions: ^{1}H 30°

flip angle of 4.5 μs, 64 number of scans, 16 dummy scans, 3 s relaxation delay, 11.5 kHz spectral width and 32 K number of points.

Diffusion measurements were performed using the double stimulated echo (DSTE) sequence (Jerschow & Muller, 1997). 0.3 ml of sample was filled into 5 mm NMR tube (WILMAD-LABGLASS SP Industries Co., Buena, NJ). A plug was inserted in the tube at the bottom and at the top of the solution (SP-PS-5, WILMAD-LABGLASS SP Industries Co., Buena, NJ) to optimize the homogeneity of the system. The data for PGSE experiments were collected with equal g^2 spacing gradient. The gradient amplitude (g) was changed from 0.718 to 34.125 G cm^{-1} in 64 constant steps. The gradient pulse length was 2.2 ms and diffusion delay time was selected in the range 80–150 ms. DOSY NMR data were processed using the Bruker software XWINNMR 3.5. For internal reliability, the diffusion of DMF-d_7 was measured and coefficient obtained (1.41×10^{-9} m^2 s^{-1}) resulted in agreement with the value reported in the literature (Holz, Mao, Seiferling, & Sacco, 1996).

3. Results and discussion

In the first set of experiments, interactions between daidzein and soy protein were studied by application of one-dimensional (1D) ^{1}H NMR spectroscopy. The overall 1D spectrum of daidzein with and without soy protein is shown in Fig. 2a. In Fig. 2b–d specific areas of the spectrum were enlarged to highlight the main chemical shift changes after the addition of soy protein to daidzein solution.

Fig. 2b shows a shifting of the signal observed for the proton on the pyranone ring of daidzein upon protein addition (2H–C). Other significant chemical shift variations of protons located on polyphenolic rings of the daidzein were not observed (Fig. 2c), indicating their minimal involvement in interactions with the protein.

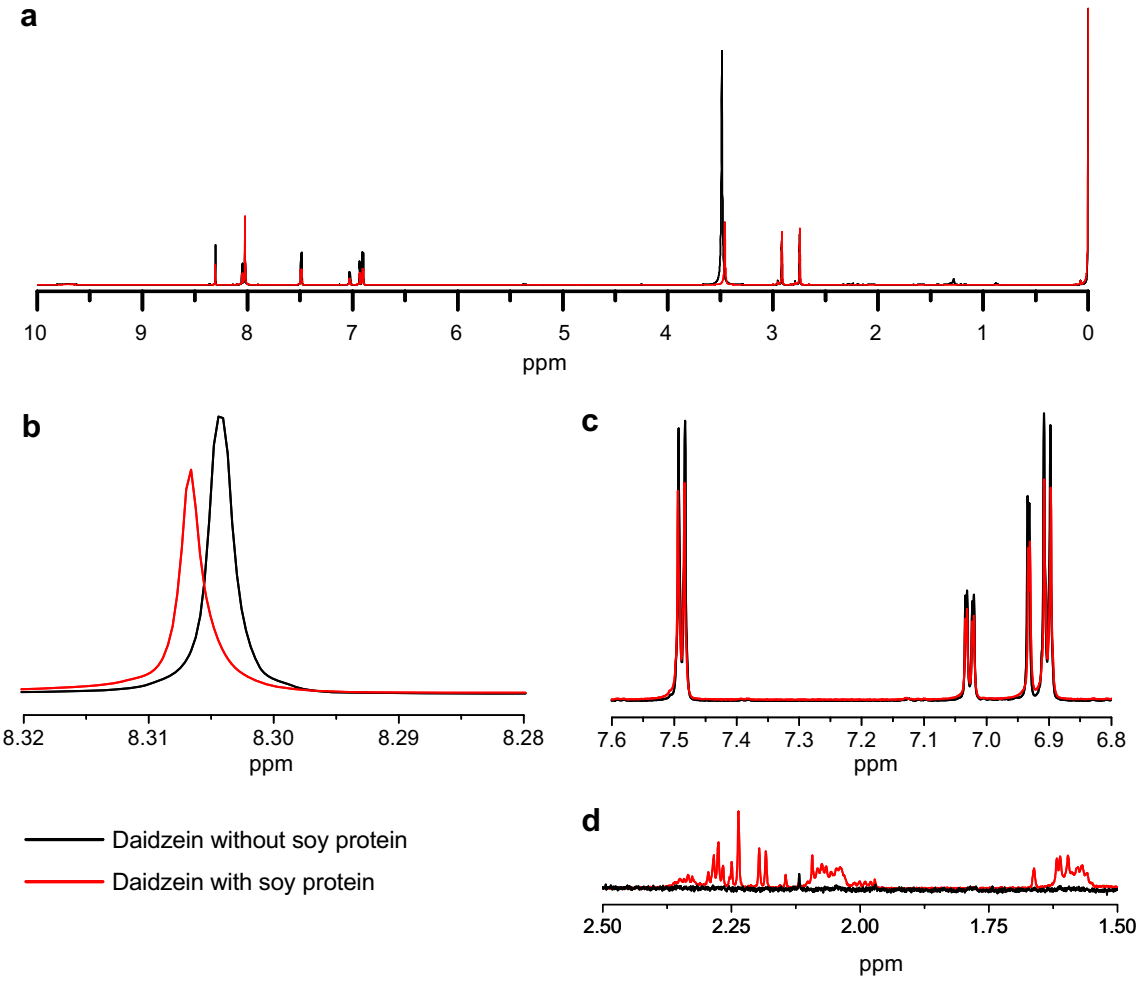

Fig. 2. ^{1}H 1D NMR spectra of daidzein with and without soy protein. 1D frames show the overlap between the two mixtures in the overall ppm scale (0–10 ppm) (a). Frame b shows the variation of chemical shift related to the hydrogen located on the pyranone ring (frame 8.28–8.32 ppm). Frame c depicts no change of chemical shifts related to other hydrogen located on the polyphenol rings (frame 6.8–7.6 ppm). Frame d shows some variation of the 1D profile due to the addition of soy protein in the high field region (1.5–2.5 ppm).

Due to proton chemical exchange, the phenolic groups of daidzein are not seen in one-dimensional ^{1}H spectra and therefore their dipolar interactions are not easy to establish; however, their contribution cannot be neglected. Recent findings indicate that hydroxyl groups of flavonoids, such as genistein, are of primary importance since they form hydrogen bonds with biomolecules to stabilize biological complexes (Bocian et al., 2006; Kozerski et al., 2003). These investigations using labeled compounds show that the hydroxyl groups at C-7 and C-4′ are likely involved in intermolecular hydrogen bondings (Kozerski et al., 2003). Due to the structural similarity of daidzein to genistein (genistein has a hydroxyl group at C-5 that is not present in daidzein), a possible explanation for the lack of variations of protons signals located on polyphenolic rings in daidzein (Fig. 2c) can be attributed to the dominant hydrogen bondings between the C-7 and C-4′ hydroxyl groups of this phytoestrogen and the soy protein, as described elsewhere (Rawel et al., 2002; Rohn et al., 2005). Since the pK_a of daidzein in DMF is 11.2 (Georgievskii, 1980), and the pH of the solutions is ~7.5, hydroxyl groups of polyphenols have no charge and therefore ionic bondings are not involved in the interactions between the soy protein and isoflavones. Additionally, an interaction between the proton located on C-2 and the soy protein may be postulated due to the chemical shift variation depicted in Fig. 2b. This shifting may suggest hydrophobic interactions between the ligand and the protein, and this hypothesis may be reinforced by the hydrophobic nature of the soy protein isolate (Tiziani & Vodovotz, 2005a, 2005b).

The shift of NMR signals may also have been affected by slight differences in the sample environments. Dimethylformamide (DMF) is a highly hygroscopic solvent which readily absorbs water. Although all precautions were taken during the sample preparation, for example DSC analysis confirmed the absence of "freezable" water in the dried protein (as described in Section 2), one can not rule out the possibility of some aqueous contamination during sample preparation for NMR experiments (~3.5 ppm Fig. 2a). Therefore, to more readily probe the intermolecular interactions between isoflavones and soy protein, a second set of experiments was carried out utilizing pulse-gradient spin-echo (PGSE) NMR.

Fig. 3 shows a low field region of a representative 2D DOSY spectrum depicting clear differences in the translational diffusion coefficients of daidzein, daidzin, and DMF. Since the analyzed isoflavones and carotenoids have fully resolved resonances, the values of translational diffusion of the individual component were calculated from PGSE experiments using 2D DOSY processing technique. Because diffusion coefficients are related to the hydrodynamic radius of a molecule or aggregate, PGSE NMR technique probes the occurrence of intermolecular interactions without being affected by small environmental changes such as water contamination.

PGSE NMR experiments were carried out on different model systems using two different procedures. In the first, the affinity of daidzein and daidzin with and without the soy protein and carotenoids was analyzed without mixing the two isoflavones (Table 1, columns A–C). In the second, the phytochemicals were dissolved in the same solution and the corresponding values of diffusion were calculated in the presence of protein and carotenoids (Table 1, columns
D–F).

The values of translational diffusion reported in Table 1, columns (A–C) depict a different trend for each of the two phytoestrogens with and without the protein: the self diffusion coefficient of daidzin shows a greater decrease as compared to daidzein. The attenuation of the daidzin resonances under the influence of the linear field gradients indicates that the affinity with the soy protein isolate is higher as compared to that of daidzein with the same protein.

In addition, carotenoids extracted from tomato juice were added to the mixtures to assess their possible effect on the interactions between isoflavones and soy protein on the basis of translational diffusion coefficients of each phytochemical component dissolved in the same DMF mixture. In both isoflavone/soy protein model systems (daidzein or daidzin plus soy proteins), the addition of carotenoids from tomato juice (mainly (all-E)-lycopene and (15Z)-phytoene) does not seem to interfere with interactions between the soy protein and the phytoestrogenic compounds (Table 1, columns A–C). Because of the lipophilic nature of lycopene and phytoene, on the basis of 1D spectrum, it is hypothesized that carotenoids do not interfere with the proton located on C-2 and the soy protein (Fig. 2b).

In the second set of PGSE NMR experiments, daidzein and the corresponding glucoside conjugate were dissolved in the same solutions and different model systems were prepared in the presence of soy protein and tomato carotenoids (Table 1, columns D–F).

In the specific case of both isoflavones dissolved in DMF (Table 2 D, Fig. 3), the differences in molecular weight between the DMF (M_W: 73.09 (g/mol)) and the two other phytochemicals (M_W of daidzein: 254.24 (g/mol), M_W of daidzin: 416.38 (g/mol)) accounts for the different self-diffusion coefficients.

The diffusion values of these two isoflavones decrease as well after addition of the soy protein (Table 1, columns D–F); the diffusion coefficient of daidzin was found to be slightly more affected as compared to daidzein and this seems to indicate stronger interactions between the glucoside isoflavone and the soy protein. It is noteworthy that the diffusion values of two isoflavones present simultaneously in the mixture with the protein (Table 1, columns D–F) are not appreciably different from the coefficients reported in Table 1, columns A–C, in which the phytoestrogens were dissolved individually with the protein. This confirms the neglectable contribute of the simultaneous

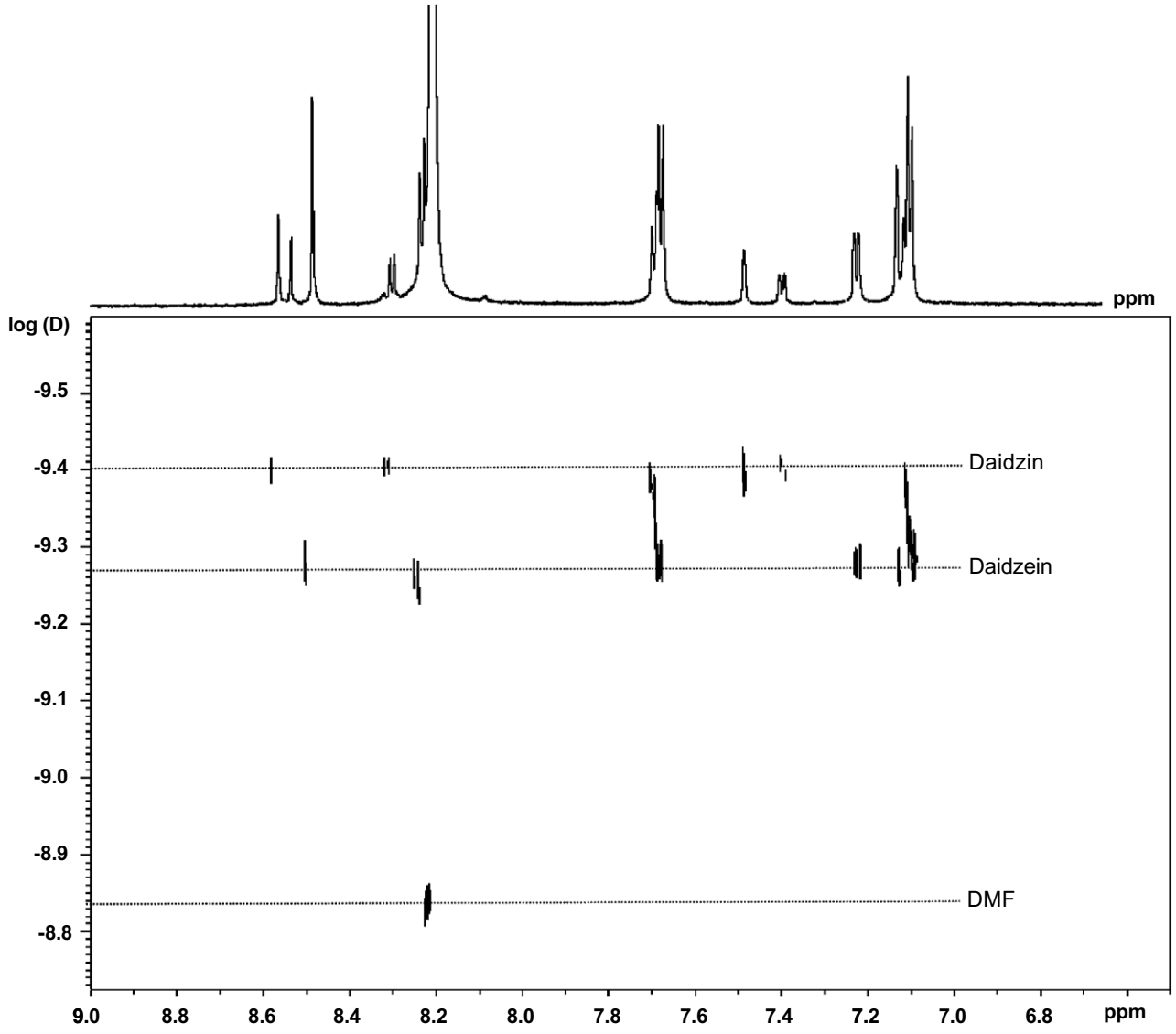

Fig. 3. Representative 2D DOSY spectrum with chemicals shift (ppm) along f_2 axis and diffusion coefficient along f_1 axis. The mixture contains standard daidzein and daidzin dissolved in DMF. Dotted lines show average diffusion coefficients of each component.

addition of both isoflavones to viscosity of different model solutions and therefore to the diffusion values of the phytochemicals.

The addition of carotenoids to the soy isoflavones/protein mixture did not affect the diffusion coefficients of the two polyphenols (Table 1, column F). Therefore, on the basis of the translational diffusion coefficient, lycopene and phytoene, the two predominant carotenoids found in the tomato juice (Tiziani et al., 2006), do not seem to impact the interaction between soy protein and isoflavones.

On the other hand, carotenoids from tomato juice extract were affected by soy protein and isoflavones. In fact, the diffusion coefficient of phytoene slightly decreased when added to the mixture of isoflavones and soy protein (Table 1, column C). Since no variation of diffusion was observed for isoflavones before and after addition of carotenoids, it is assumed that phytoene interacts with the soy protein and not with isoflavones. The translational diffusion of (all-E)-lycopene did not show a noticeable change with and without the presence of other biomolecules (Table 1, columns A, C, and F). The two carotenoids were also dissolved with soy protein isolates and not with isoflavones (data not shown) and the diffusion values were found to be similar to the coefficients reported in Table 1, column F, therefore no interaction between the two classes of phytochemicals was detected, as expected.

The self-diffusion of daidzein after addition of soy protein with and without daidzin did not show appreciable changes (Table 1, columns B and D) suggesting that soy protein has enough sites available to interact with both isoflavones and that the interactions of protein and isoflavones are dominant in this system.

The results of one-dimensional ^{1}H NMR and two-dimensional 2D DOSY studies of phytochemical model

Table 1
Diffusion coefficient ($m^2 s^{-1}$) of isoflavones (daidzein and daidzin) and carotenoids (phytoene and lycopene) calculated for different model systems (A, B, C, D, E, F) obtained by 2D DOSY NMR

Phytochemical	Diffusion coefficient ($\times 10^{-10}$ $m^2 s^{-1}$)					
	A	B	C	D	E	F
Daidzein[§]	5.37	4.92	4.92	5.31	4.90	4.89
Daidzin[§]	3.94	3.36	3.36	3.90	3.36	3.36
(15Z)-phytoene[*]	2.72	–	2.62	–	–	2.61
(all-E)-lycopene[*]	2.55	–	2.53	–	–	2.52

A: Phytochemical standard[§] or extracted[*].

B: Daidzein or daidzin + soy protein.

C: Daidzein or daidzin + soy protein + carotenoid extract.

D: Daidzein + daidzin.

E: Daidzein + daidzin + soy protein.

F: Daidzein + daidzin + soy protein + carotenoid extract.

systems indicate that the interactions between the daidzin and the soy protein isolate are stronger as compared to the aglycone isoflavone dissolved in the same model system. This and previous studies suggest that the major contribution of intermolecular hydrogen bonding from hydroxyl groups and the minor one from the proton located on the pyranone ring (2H–C) of isoflavones are responsible for the interactions between the isoflavones and the soy protein isolate. The addition of carotenoids to soy isoflavones/protein system does not seem to interfere with the interactions between the polyphenols and the protein. These results are significant with regard to further experiments planned to understand the role and the impact of the individual phytochemical on the bioaccessibility and bioavailability of functional foods.

Acknowledgements

We wish to acknowledge Dr. Charles Cottrell and Dr. Chunhua Yuan of the Campus Chemical Instrument Center of The Ohio State University for their kind technical help for NMR experiments.

References

Arrese, E. L., Sorgentini, D. A., Wagner, J. R., & Anon, M. C. (1991). Electrophoretic, solubility, and functional-properties of commercial soy protein isolates. *Journal of Agricultural And Food Chemistry, 39*(6), 1029–1032.

Asano, K., Shinagawa, K., & Hashimoto, N. (1982). Characterization of haze-forming proteins of beer and their roles in chill haze formation. *Journal of the American Society of Brewing Chemists, 40*(4), 147–154.

Barnes, S., Kirk, M., & Coward, L. (1994). Isoflavones and their conjugates in soy foods - Extraction conditions and analysis by Hplc mass-spectrometry. *Journal of Agricultural and Food Chemistry, 42*(11), 2466–2474.

Baxter, N. J., Lilley, T. H., Haslam, E., & Williamson, M. P. (1997). Multiple interactions between polyphenols and a salivary proline-rich protein repeat result in complexation and precipitation. *Biochemistry, 36*(18), 5566–5577.

Bianco, A., Chiacchio, U., Rescifina, A., Romeo, G., & Uccella, N. (1997). Biomimetic supramolecular biophenol-carbohydrate and biophenol-protein models by NMR experiments. *Journal of Agricultural and Food Chemistry, 45*(11), 4281–4285.

Bocian, W., Kawecki, R., Bednarek, E., Sitkowski, J., Ulkowska, A., & Kozerski, L. (2006). Interaction of flavonoid topoisomerase I and II inhibitors with DNA oligomers. *New Journal of Chemistry, 30*(3), 467–472.

Brooks, J. R., & Morr, C. V. (1985). Current aspects of soy protein fractionation and nomenclature. *Journal of the American Oil Chemists Society, 62*(9), 1347–1354.

Cassidy, A., Hanley, B., & Lamuela-Raventos, R. M. (2000). Isoflavones, lignans and stilbenes – Origins, metabolism and potential importance to human health. *Journal of the Science of Food and Agriculture, 80*(7), 1044–1062.

Clinton, S. K. (1998). Lycopene: Chemistry, biology, and implications for human health and disease. *Nutrition Reviews, 56*(2), 35–51.

Dhaubhadel, S., McGarvey, B. D., Williams, R., & Gijzen, M. (2003). Isoflavonoid biosynthesis and accumulation in developing soybean seeds. *Plant Molecular Biology, 53*(6), 733–743.

FDA (1999). Food labeling: Health claims; soy protein and coronary heart disease. Food and Drug Administration, HHS. Final rule. *Federal Register, 64*(206), 57700–57733.

Gann, P. H., Ma, J., Giovannucci, E., Willett, W., Sacks, F. M., Hennekens, C. H., et al. (1999). Lower prostate cancer risk in men with elevated plasma lycopene levels: Results of a prospective analysis. *Cancer Research, 59*(6), 1225–1230.

Georgievskii, V. P. (1980). Acid properties of flavonoid compounds and choice of a solvent for carrying out potentiometric analysis. *Khimiya Prirodnykh Soedinenii, 2*, 180–186.

Giovannucci, E. (2002). A review of epidemiologic studies of tomatoes, lycopene, and prostate cancer. *Experimental Biology and Medicine, 227*(10), 852–859.

Gounarides, J. S., Chen, A. D., & Shapiro, M. J. (1999). Nuclear magnetic resonance chromatography: Applications of pulse field gradient diffusion NMR to mixture analysis and ligand–receptor interactions. *Journal of Chromatography B – Analytical Technologies in the Biomedical and Life Sciences, 725*(1), 79–90.

Hasler, C. M., Bloch, A. S., Thomson, C. A., Enrione, E., & Manning, C. (2004). Position of the American Dietetic Association: Functional foods. *Journal of the American Dietetic Association, 104*(5), 814–826.

Holz, M., Mao, X. A., Seiferling, D., & Sacco, A. (1996). Experimental study of dynamic isotope effects in molecular liquids: Detection of translation–rotation coupling. *Journal of Chemical Physics, 104*(2), 669–679.

Jerschow, A., & Muller, N. (1997). Suppression of convection artifacts in stimulated-echo diffusion experiments. Double-stimulated-echo experiments. *Journal of Magnetic Resonance, 125*(2), 372–375.

Johnson, C. S. (1999). Diffusion ordered nuclear magnetic resonance spectroscopy: Principles and applications. *Progress in Nuclear Magnetic Resonance Spectroscopy, 34*(3–4), 203–256.

Johnson, I., & Williamson, G. (2003). *Phytochemical functional foods*. Cambridge, England: CRC Press.

Khachk, F., Beecher, G. R., Goli, M. B., & Lusby, W. R. (1992). Separation and quantification of carotenoids in foods. *Methods in Enzymology, 213*, 347–359.

Kozerski, L., Kamienski, B., Kawecki, R., Urbanczyk-Lipkowska, Z., Bocian, W., Bednarek, E., et al. (2003). Solution and solid state C-13 NMR and X-ray studies of genistein complexes with amines. Potential biological function of the C-7, and C-4′-OH groups. *Organic and Biomolecular Chemistry, 1*(20), 3578–3585.

Kroll, N. G., Rawel, H. M., & Rohn, S. (2003). Reactions of plant phenolics with food proteins and enzymes under special consideration of covalent bonds. *Food Science and Technology Research, 9*(3), 205–218.

Lucas, L. H., & Larive, C. K. (2004). Measuring ligand–protein binding using NMR diffusion experiments. *Concepts in Magnetic Resonance Part A, 20A*(1), 24–41.

Morris, K. F., & Johnson, C. S. (1992). Diffusion-ordered 2-dimensional nuclear-magnetic-resonance spectroscopy. *Journal of the American Chemical Society, 114*(8), 3139–3141.

Morris, K. F., Stilbs, P., & Johnson, C. S. (1994). Analysis of mixtures based on molecular-size and hydrophobicity by means of diffusion-ordered 2D NMR. *Analytical Chemistry, 66*(2), 211–215.

Nielsen, N. C. (1985). The structure and complexity of the 11S polypeptides in soybeans. *Journal of the American Oil Chemists Society, 62*(12), 1680–1686.

Price, W. S. (1998). Pulsed-field gradient nuclear magnetic resonance as a tool for studying translational diffusion: Part II. Experimental aspects. *Concepts in Magnetic Resonance, 10*(4), 197–237.

Puppo, M. C., & Añón, M. C. (1999). Soybean protein dispersions at acid pH. Thermal and rheological properties. *Journal of Food Science, 64*(1), 50–56.

Rawel, H. M., Czajka, D., Rohn, S., & Kroll, J. (2002). Interactions of different phenolic acids and flavonoids with soy proteins. *International Journal of Biological Macromolecules, 30*(3–4), 137–150.

Rawel, H. M., Kroll, J., & Hohl, U. C. (2001). Model studies on reactions of plant phenols with whey proteins. *Nahrung-Food, 45*(2), 72–81.

Rochfort, S. (2005). Metabolomics reviewed: A new "Omics" platform technology for systems biology and implications for natural products research. *Journal of Natural Products, 68*(12), 1813–1820.

Rohn, S., Rawel, H. M., Rober, M., & Kroll, J. (2005). Reactions with phenolic substances can induce changes in some physico-chemical properties and activities of bromelain – The consequences for supplementary food products. *International Journal of Food Science and Technology, 40*(7), 771–782.

Siebert, K. J. (1999). Effects of protein–polyphenol interactions on beverage haze. Stabilization and analysis. *Journal of Agricultural and Food Chemistry, 47*(2), 353–362.

Steinmetz, K. A., & Potter, J. D. (1996). Vegetables, fruit, and cancer prevention: A review. *Journal of the American Dietetic Association, 96*(10), 1027–1039.

Tiziani, S., Schwartz, S. J., & Vodovotz, Y. (2006). Profiling of carotenoids in tomato juice by one- and two-dimensional NMR. *Journal of Agricultural and Food Chemistry, 54*(16), 6094–6100.

Tiziani, S., & Vodovotz, Y. (2005a). Rheological characterization of a novel functional food: Tomato juice with soy germ. *Journal of Agricultural and Food Chemistry, 53*(18), 7267–7273.

Tiziani, S., & Vodovotz, Y. (2005b). Rheological effects of soy protein addition to tomato juice. *Food Hydrocolloids, 19*(1), 45–52.

Tsangalis, D., Ashton, J. F., Stojanovska, L., Wilcox, G., & Shah, N. P. (2004). Development of an isoflavone aglycone-enriched soymilk using soy germ, soy protein isolate and bifidobacteria. *Food Research International, 37*(4), 301–312.