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Short-term stability of soy isoflavones extracts: Sample conservation aspects

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Abstract

Short-term storage stability of soy isoflavones was determined by evaluating the effects of storage duration, temperature, UV–Vis light and vial head space on individual isoflavone concentration. Concentration of isoflavones in ethanol-water extracts and standard solutions stored with temperatures between −20 and 10 °C remains unchanged up to one week. Degradation of malonyl isoflavones is affected by storage duration, temperature, incident UV–Vis light and vial head space. Glucoside isoflavones were not sensitive to the experimental storage conditions. Daidzein and glycitein were the only aglucones sensitive to UV–Vis light. Isoflavones must be kept at temperatures lower than 10 °C and protected from light. Using these conditions, the extracts can be stored up to one week with no significant degradation of isoflavones allowing better planning of routine analysis of large number of samples.

Keywords: Isoflavones; Stability; Soybeans; Storage; Sample conservation; HPLC

1. Introduction

Soy foods are widely promoted and consumed based on a supposed relationship between its consumption and beneficial health effects in humans including chemoprevention of breast and prostate cancer, osteoporosis, and arteriosclerosis. Evidence provided by epidemiological studies showing a lower incidence of these health conditions in countries like Japan and China, which have high soy consumption, is pointed out as the basis of this relationship (Adlercreutz & Mazur, 1997; Barnes, Peterson, Grubbs, & Setchell, 1994; Lee et al., 1991). Since soybeans contain high amounts of isoflavones (0.1–5.0 mg of total isoflavones per gram) (Coward, Barnes, Setchell, & Barnes, 1993) which have shown several in vitro and in vivo properties consistent with these possible effects in humans, an association between isoflavone intake and health protective effects of soybeans was forth seen.

Isoflavones are a subclass of flavanoids and are also called phytoestrogen compounds due to its structural similarity with human hormone estradiol. The main isoflavones found in soybeans are genistin, daidzin, glycitin and their respective acetyl, malonyl and aglucone forms (Fig. 1) (Naim, Gestetner, Zilkah, Birk, & Bondi, 1974; Walter, 1941; Walz, 1931). In soybeans, the conjugates of genistein, daidzein and glycitein are found in an approximately ratio of 6:3:1, respectively, although the isoflavone contents is influenced by genetics, crop year, and growth location (Wang & Murphy, 1994).

Many mechanisms of action have been proposed for isoflavone prevention of diseases, including estrogenic/antiestrogenic activity, antiproliferation, induction of cell-cycle arrest and apoptosis, prevention of oxidation, regulation of the host immune system, and changes in cellular signaling (Adlercreutz, 1999; Birt, Hendrich, &

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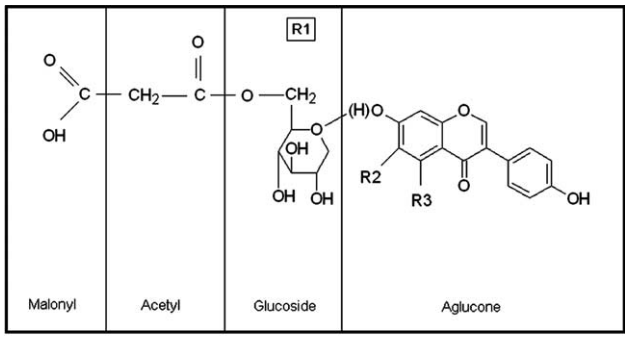

Isoflavone	Symbol	R1	R2	R3
Genistein	Ge	H	H	OH
Daidzein	De	H	H	H
Glycitein	Gle	H	OCH_3	H
Genistin	Gi	$C_6O_5H_{11}$	H	OH
Daidzin	Di	$C_6O_5H_{11}$	H	H
Glycitin	Gly	$C_6O_5H_{11}$	OCH_3	H
Acetyl-genistin	AGi	$C_6O_5H_{11}+COCH_3$	H	OH
Acetyl-daidzin	ADi	$C_6O_5H_{11}+COCH_3$	H	H
Acetyl-glycitin	AGly	$C_6O_5H_{11}+COCH_3$	OCH_3	H
Malonyl-genistin	MGi	$C_6O_5H_{11}+COCH_2COOH$	H	OH
Malonyl-daidzin	MDi	$C_6O_5H_{11}+COCH_2COOH$	H	H
Malonyl-glycitin	MGly	$C_6O_5H_{11}+COCH_2COOH$	OCH_3	H

Fig. 1. Chemical structure of soy isoflavones.

Wang, 2001; Lissin & Cooke, 2000; Setchell & Adlercreutz, 1988). The actual mechanisms by which the isoflavones act in the human organism have not been fully established and metabolism may play an important role.

Since some studies have shown that the biological activity of isoflavones may depend on the type of soy food and its processing and storage conditions (Singletary, Faller, Li, & Mahungu, 2000), several studies about the stability of isoflavones during processing and storage have been reported in the last few years. Decarboxilation of malonate to acetate and de-esterefication of malonate or acetate to underivatized glucoside as well as generation of aglucone and acetyl isoflavones, is known to take place depending upon extraction, processing and storage conditions (Coward, Smith, Kirk, & Barnes, 1998; Grün et al., 2001; Jackson et al., 2002; Kao, Su, & Lee, 2004; Mahungu et al., 1999; Simonne et al., 2000; Wang & Murphy, 1996).

Amazingly, only a few have made observations about the stability of the extracts and sample conservation aspects. This is one of the most important aspects in the isoflavone analysis for exact quantification of these compounds in foods since some isoflavones are sensitive to degradation.

Recently, Rijke, Zafra-Gómez, Ariese, and Brinkman (2001) studied the stability of isoflavone red clover extracts for 1–2 weeks and observed that they can be stored for at least 1–2 weeks at −20 °C without loss of biochanin and formononetin malonates. However, if the sample is kept at room temperature and also if it is stored dry at −20 °C, degradation starts to take place almost immediately. Curiously, they also observed that in liquid chromatagraphy (LC) separated fractions, these malonates are most stable when stored at low temperature after evaporation to dryness. The report did not include most common malonyl isoflavones present in soybeans.

The most susceptible to degradation isoflavones are the malonyl forms. Murphy et al. (1997), for example, reported a conversion rate of 0.2–0.3 mol% per hour of malonyl forms to glucosides in soy isoflavone extracts at room temperature. Obviously prompt analysis of the extracts after extraction is necessary to minimize degradation of malonyl isoflavones. Another strategy used to minimize degradation is the maintenance of auto sampler at low temperatures (4–5 °C) (Coward et al., 1998). Although, these procedures can overcome potential analysis errors it would be very useful to evaluate the stability of soy isoflavones extracts under common used storage conditions to allow better planning of routine analysis of large number of samples. Thus, our objective in this work was to evaluate short-term storage stability of soy isoflavones in extracts and standard solutions.

2. Materials and methods

2.1. Chemicals and solvents

Ethanol and methanol (Panreac, Barcelona, Spain) used in the experiments and analysis were high performance liquid chromatography (HPLC) grade. Water was supplied by a Milli-Q water purifier system from Milipore (Bedford, MA, USA). Standards of genistein (Ge), daidzein (De) and glycitein (Gle) were obtained from Sigma Chemical Co. (St. Luis, MO, USA) while standards of genistin (Gi), daidzin (Di), glycitin (Gly), malonyl daidzin (MDi), malonyl glycitin (MGly) and malonyl genistin (MGi) were obtained from LC Labs (Woburn, MA, USA).

2.2. Extraction of soy isoflavones

Extractions of freeze-dried soybeans were carried out in an ultrasonic bath of 360W (J.P. Selecta, Barcelona, Spain) using ethanol 50% in water as extracting solvent. Three grams of freeze-dried soybeans were extracted by 25 mL of ethanol at 60 °C for 20 min (Rostagno, Palma, & Barroso, 2003). Extractions were performed at con-

stant temperature by means of a temperature controller (Digit Cool, J.P. Selecta, Barcelona, Spain) coupled to the ultrasonic bath. Several extractions were performed to obtain a large extract volume.

2.3. Samples

Approximately one liter of the extract was filtered through filter paper, freeze-dried and re-constituted in ethanol 50% in water. The resulting extract was used as reference extract for the stability studies. It was divided into several aliquots and then filtered through a 0.45 μm nylon syringe filter (Millex-HN, Ireland) directly into the HPLC plastic shell conical vials of 700 μL (8 × 40 mm, Waters, Milford, MA, USA). The major constituents in the extract were Gi, MGi, Di, MDi, Gly and MGly. Concentrations of isoflavones present in the resulting extract are presented in Table 1. Aglucone isoflavones were not detected in the extract; therefore stability of Ge, De and Gle was evaluated using standard solutions (ethanol 50% in water). Concentrations (μM) of Ge, De and Gle in the standard solution used in the experiments are also shown in Table 1.

2.4. Experimental conditions

Experiments to determine the stability of isoflavone extracts from soybeans and standard solutions were performed inside a climatic chamber with temperature and UV–Vis light control (Sociedad Española para el Control de Calidad e Instrumentación S.L, Barcelona, Spain). The climatic chamber provides controlled ambient conditions with uniformity in both temperature and UV–Vis light incidence. It is equipped with a 1500 W xenon lamp providing a chromatic temperature of 5800 K in the 240–680 nm range. In that way, several ambient conditions for storage of samples inside HPLC vials were tested. Experimental conditions inside the climatic chamber were: temperature (10, 25 and 40 °C); head space 0% (full vials) and 50% (half full vials); incident UV–Vis light (0% and 100%) and storage period (1–7 days). The vials were shaken and rotated inside the climatic chamber four times per day. For temperatures of 5 °C, samples were stored inside a household refrigerator and for temperatures of −20 °C inside a freezer. All experiments were performed in triplicate.

2.5. High performance liquid chromatography

The HPLC analysis of the isoflavones was performed on an RP-18 (LiChrosphere 100, 5 μm, Merck, Darmstad, Germany) reverse-phase column using a HPLC system (Waters) composed by a auto sampler (717 plus), pump controller (600S), pump (616), and a photodiode array detector (996). UV detection was performed at a wavelength of 254 nm and the injection volume was 10 μL. The mobile phase used for analysis was solvent A: 0.1% acetic acid solution and solvent B: acidified methanol (0.1% acetic acid). A flow rate of 0.5 mL/min under the initial condition of 80:20 (A:B) was brought to 30% B in 15 min, to 45% B in 10 min, to 50% B in 5 min, and to 80% B in 10 min, all with a linear gradient. Finally, 100% B was used for 10 min to wash the column. The software for the control of the equipment, acquisition and data treatment, was Millennium (version 3.10). Di, Gly, Gi, MDi, MGly, MGi, De, Gle and Ge were separated in sequence with the gradient elution. The identity of each separated compounds was assigned by comparison of retention times, UV–Vis spectra and mass spectra, besides of co-chromatography with the authentic standards. Quantification was carried out by integration of the peak areas using the external standardisation method. The correlation coefficient of each calibration curve reached 0.999. Detection limits (mg/L) for Di, Gly, Gi, MDi, MGly, MGi, De, Gle and Ge, were 0.485, 0.390, 0.480, 0.430, 0.353, 0.488 and 0.453, respectively. Quantification limits (mg/L) for Di, Gly, Gi, MDi, MGly, MGi, De, Gle and Ge, were 1.617, 1.301, 1.600, 1.434, 1.177, 1.627 and 1.510, respectively. Detection and quantification limits were calculated using ALAMIN software (Campana, Rodrıguez, Barrero, Ceba, & Fernández, 1997). To ensure that no alteration of the extract have taken place during the experiments, daily analysis of the extract were performed and peak areas of all isoflavones compared. Continuous analysis of the reference extract and standard solutions for 24 h revealed an overall peak area variability lower than 2.5% and therefore analysis were performed within this period.

2.6. High performance liquid chromatography–mass spectrometry

The HPLC–MS analyses of the extracts were performed in a Finnigan LCQTM coupled LC–MS system,

Table 1
Isoflavone concentration (μM ± SD) in the reference extract (malonyl glucosides and glucosides) and standard solutions (aglucones) used in the experiments

Isoflavone concentration (μM)								
MGi	Gi	MDi	Di	MGly	Gly	Ge	De	Gle
56.21 ± 1.23	61.24 ± 0.98	10.97 ± 0.50	11.38 ± 0.51	5.91 ± 0.18	9.01 ± 0.37	15.21	6.61	8.32

of Finnigan MAT (Thermo Electron Co., San Jose, USA). This equipment is fitted with a Spectra SYSTEM 2000 model gradient pump (Thermo Separation Products, Fremont, USA) and a mass detector (model LCQ) that consists of an electrospray interface and an ion trap mass analyser. The same chromatographic elution and column as in HPLC–PDA analyses were used. Xcalibur (version 1.2) software was used for the control of the equipment, acquisition and data treatment. The injection volume was 100 μL. The interface conditions were: positive ionization mode, temperature of the capillary: 220 °C, spray voltage: 4.6 kV, capillary voltage: −5 V, sheath gas flow: 80 (arbitrary units) and auxiliary gas flow: 10 (arbitrary units). EPI-MS spectra were acquired in the *m/z* range of 200–600.

3. Results and discussion

Changes in the concentration of each isoflavone were studied over a period of 7 days. Fig. 2 shows individual isoflavone concentration during the experimental period under different temperatures (10, 25 and 40 °C). Temperatures below 60 °C were checked as it has been already proved that working over 60 °C malonyl conjugates are easily degraded. All concentrations in Fig. 2 are relative to the amount of isoflavones found in the reference extract (100%) and to vials stored with 0% head space and without incident UV–Vis light. Table 2 shows the relative concentration of isoflavones stored at different conditions at the end of the 7 days period. All concentrations in Table 2 are also relative to the amount found in the reference extract (100%).

The results displayed in Fig. 2 shows that storage duration affected the isoflavone profile of the sample but was dependent of the storage temperature. At lower temperatures (10 °C) individual isoflavone concentration remained constant while at higher temperatures it was observed a steady and daily decrease in the concentration of malonyl isoflavones. Concentration of the respective glucoside constantly increased at the same rate while concentration of aglucones remained unchanged. Degradation of malonyl forms producing glucosyl forms has been already reported in the literature (Coward et al., 1998; Mahungu et al., 1999). Therefore, if the goal of the analysis is the determination of individual isoflavones including malonyl forms, it can be concluded that maximum storage duration is fully conditioned by the storage temperature.

Temperature is the major problem regarding the stability of isoflavones extracts during food processing and it can be expected to be the most important factor in sample handling and conservation. Individual isoflavone concentration remained constant (overall variability lower than 5%) when extracts and standard solutions were stored at −20 °C for 30 days and under refrigeration (5–6 °C) for 7 days, indicating an adequate storage condition. The effect of temperature on isoflavone concentration is evident when comparing the extracts stored at temperatures between 10 and 40 °C (Fig. 2).

As can be seen, concentration of isoflavones stored at 10 °C for 7 days remained unchanged indicating that extracts and standard solutions can be stored under these conditions.

At 25 °C, a clear effect of the temperature in the isoflavone profile of the sample was observed. Concentration of MGi, MDi and MGly decreased with a correspondent increase in the concentration of Gi, Di and Gly, indicating conversion of malonyl isoflavones to the respective glucoside. Conversion rates (μM/h) at 25 °C for MGi, MDi and MGly to Gi, Di and Gly were 0.117, 0.018 and 0.010, respectively. MGi, MDi and MGly concentration at the end of the 7 days were, respectively, 65.14%, 71.89% and 72.40% relative to their concentration in the reference extract. Although no direct information about the stability of the glucosyl forms of the isoflavones can be drawn from studies with extracts, where all isoflavones are present, careful observation of the concentration of its degradation products, their respective aglucones, can provide valuable information. If degradation of isoflavone glucosides is taking place, it can be expected to detect an increase in the concentration of the respective aglucone form in the extract. Since concentration of isoflavone aglucones was constant during the 7 days period at 25 °C, it can be assumed that isoflavone glucosides are stable under these conditions.

At 40 °C there is an important change in the isoflavone profile of the sample as can be seen in Fig. 2, mainly due to the significantly increased magnitude of malonyl isoflavones degradation. The analysis of the extracts maintained at 40 °C showed a much faster conversion rate of malonyl isoflavones to the respective glucoside than at 25 °C. Malonyl isoflavone concentration decreased sharply and glucoside concentration increased at the same rate. Conversion rates (μM/h) of MGi, MDi and MGly to Gi, Di and Gly at 40 °C were, 0.236, 0.038 and 0.021, respectively. MGi, MDi and MGly concentration at the end of the 7 days were, respectively, 29.60%, 42.23% and 40.93% relative to their concentration in the reference extract. So, increasing temperature from 25 to 40 °C degradation of malonyl forms increased by two.

In order to verify the conversion from malonyl to glucosidic form, total amounts of each isoflavone conjugates were calculated for all temperatures. Table 3 shows that there was no difference between total amounts of Gi conjugates (MGi + Gi), Di conjugates (MDi + Di) and Gly conjugates (MGly + Gly) as well as total isoflavone concentration. It can be drawn from these results that decrease in concentration of malonyl isoflavones and increase in glucoside concentration is

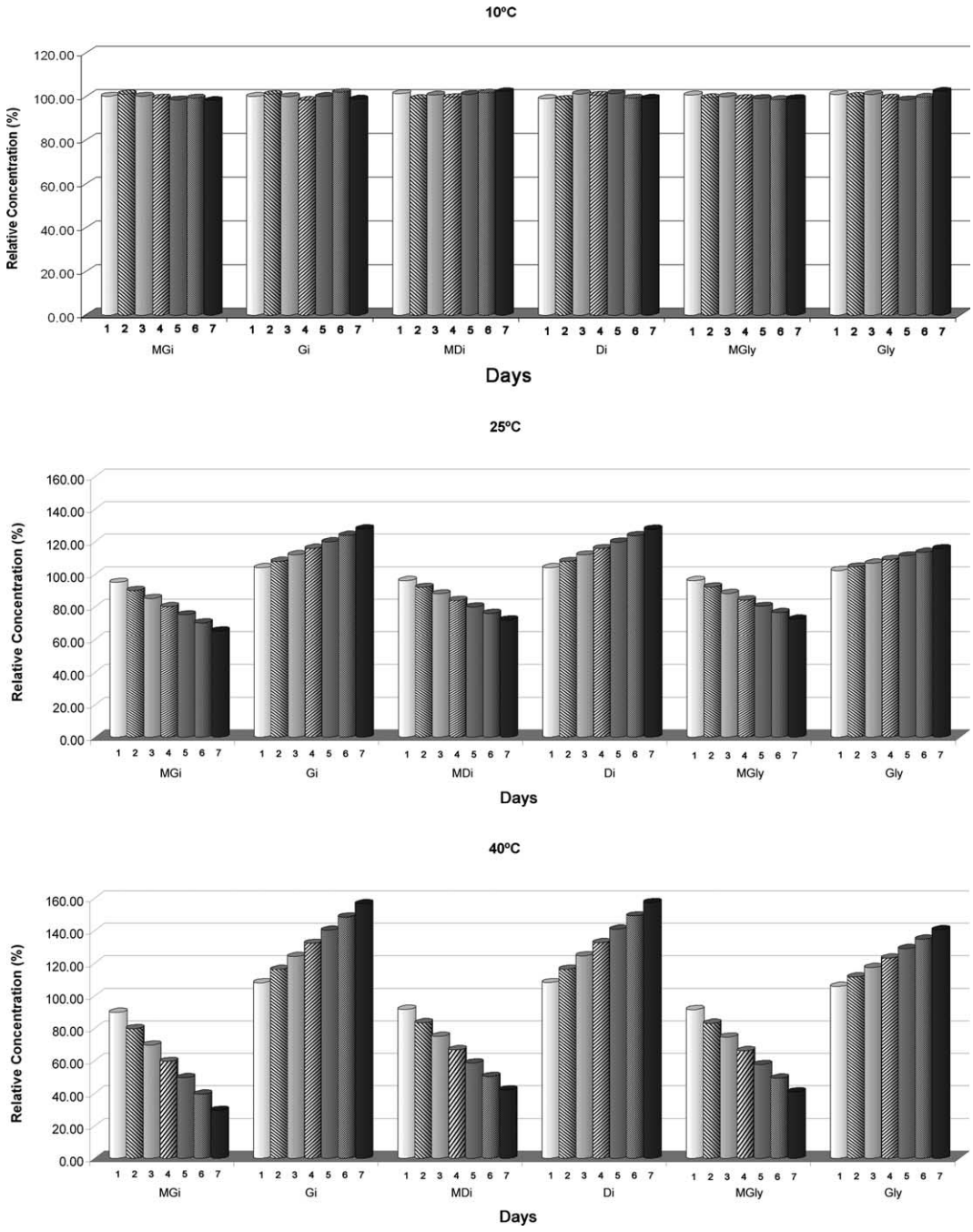

Fig. 2. Effect of temperature on the concentration (μM) of tested isoflavones in the soy extract stored inside the climatic chamber for 7 days.

exclusively due to conversion between these isoflavone forms. Concentration of isoflavone aglucones remained unchanged at 40 °C, as at 25 °C, and thus it can be considered that degradation of glucosides is not taking place at this temperature.

The UV–Vis light influence on isoflavone profile was also assessed in the study. Higher conversion rates of malonyl isoflavones to glucosyl forms were found in the vials kept with incident UV–Vis light than in vials kept without incident UV–Vis light. Malonyl isoflavones concentration in the extracts stored at 25 °C with incident UV light was, in average, 9.95% lower than in extracts stored in without incident UV light. Isoflavone glucosides were not affected by the UV–Vis light under all experimental conditions.

Concentration of aglucones stored without incident UV–Vis light at 25 °C remained unchanged. However, the situation changed radically when the sample was stored with incident UV–Vis light and a clear effect on the concentration of some aglucones was observed.

Table 2
Relative isoflavone concentration (relative % ± RSD) in the extract (malonyl glucosides and glucosides) and standard solutions (aglucones) stored for 7 days

UV light	Head space (%)	Temperature	Relative isoflavone concentration (relative % ± RSD)								
		(° C)	MGi	Gi	MDi	Di	MGly	Gly	Ge	De	Gle
No	0	−20	100.54 ± 1.94	99.31 ± 2.02	101.51 ± 2.12	101.09 ± 1.86	98.95 ± 1.90	100.90 ± 1.56	99.71 ± 1.05	100.47 ± 1.01	100.39 ± 0.95
		5	101.13 ± 2.10	100.69 ± 2.47	99.12 ± 2.31	100.73 ± 1.98	100.66 ± 1.96	99.31 ± 2.09	100.39 ± 0.99	99.68 ± 0.94	100.00 ± 0.89
No	0	10	99.42 ± 2.08	98.70 ± 2.76	99.36 ± 2.06	99.59 ± 2.13	99.48 ± 2.44	100.22 ± 1.58	100.45 ± 0.95	100.36 ± 1.05	99.85 ± 0.93
		25	74.54 ± 2.34	125.21 ± 2.36	81.22 ± 2.26	116.52 ± 2.30	83.08 ± 2.63	107.77 ± 2.78	100.19 ± 1.01	99.76 ± 0.98	100.92 ± 0.95
		40	39.60 ± 2.76	157.36 ± 1.82	60.62 ± 2.81	135.59 ± 2.11	47.55 ± 2.27	132.74 ± 2.26	100.32 ± 0.97	100.48 ± 0.97	100.15 ± 1.02
Yes	0	10	99.22 ± 2.41	100.98 ± 2.93	101.19 ± 2.91	99.21 ± 2.43	99.15 ± 2.58	101.33 ± 2.83	99.68 ± 0.95	99.88 ± 1.03	100.31 ± 0.96
		25	65.15 ± 2.73	134.23 ± 2.02	71.92 ± 2.18	127.77 ± 2.20	72.42 ± 2.74	115.76 ± 2.36	100.39 ± 0.96	88.17 ± 1.00	85.38 ± 0.97
		40	29.62 ± 2.50	164.39 ± 1.75	42.21 ± 2.54	157.47 ± 3.10	40.95 ± 2.15	140.73 ± 2.31	99.87 ± 1.05	76.94 ± 0.95	78.62 ± 0.99
No	50	10	100.34 ± 2.51	99.65 ± 1.89	100.99 ± 2.10	99.31 ± 1.54	101.01 ± 1.69	100.69 ± 1.87	100.19 ± 0.94	100.24 ± 0.93	100.32 ± 0.94
		25	72.79 ± 2.03	126.68 ± 2.18	79.88 ± 2.46	118.71 ± 2.17	80.93 ± 2.24	109.31 ± 2.96	100.58 ± 0.94	100.06 ± 0.98	100.63 ± 0.96
		40	35.92 ± 2.01	161.95 ± 2.68	56.22 ± 2.47	140.14 ± 1.98	44.51 ± 2.61	135.73 ± 2.00	100.05 ± 0.99	100.36 ± 0.96	100.46 ± 1.04
Yes	50	10	99.14 ± 2.01	100.35 ± 1.69	99.84 ± 2.36	101.06 ± 2.74	99.93 ± 2.40	100.96 ± 1.29	100.19 ± 0.97	100.60 ± 1.03	100.31 ± 0.98
		25	62.02 ± 2.41	132.54 ± 1.79	70.30 ± 2.09	125.94 ± 2.43	70.36 ± 1.86	114.30 ± 2.52	99.94 ± 0.96	88.77 ± 0.98	84.62 ± 0.99
		40	26.24 ± 2.46	167.58 ± 2.37	39.31 ± 2.03	165.67 ± 1.96	37.13 ± 2.78	143.92 ± 3.14	100.21 ± 0.90	76.46 ± 0.97	78.92 ± 0.93

Table 3
Total amount (μM ± SD) of genistin (MGi + Gi), daidzin (MDi + Di) and glycitin (MGly + Gly) conjugates during storage of the extract

Temperature (° C)	Total isoflavone conjugate concentration (μM ± SD)			
	MGi + Gi	MDi + Di	MGly + Gly	TOTAL
10	117.36 ± 3.12	22.25 ± 1.01	15.01 ± 0.63	154.61
25	118.66 ± 3.05	22.07 ± 0.95	14.74 ± 0.56	155.47
40	117.98 ± 2.97	22.37 ± 0.99	15.15 ± 0.51	155.50

Values are relative to the whole experimental period under all conditions.

While concentration of Ge remained constant, De and Gle concentrations decreased significantly, especially De.

At 40 °C, malonyl isoflavone concentration of extracts maintained with incident UV–Vis light were, in average, 11.45% lower than in extracts maintained without UV–Vis light. This is an indication that the UV–Vis light effect on malonyl isoflavone concentration is not highly influenced by the temperature increase since differences between 25 and 40 °C are not significant. The higher temperature increased the degradation of De and Gle in the vials kept with incident UV–Vis light. It was also observed that degradation of De was higher than Gle as in 25 °C.

Exposure of samples for long periods of time to UV light coupled to high temperatures can have an important impact on the isoflavone profile. This information is very useful since long static extraction methods, as for example soaking for 24 or 48 h, can overestimate glucoside concentration and underestimate malonyl glucoside concentration since the extracts will be exposed to light at room temperature, which could be assumed to be 25 °C. This makes interesting the development of fast analysis and extraction methods that are protected from the ambient light like supercritical fluid extraction (Rostagno, Araujo, & Sandi, 2002) and pressurized liquid extraction (Rostagno, Palma, & Barroso, 2004). It also indicates that long extraction methods should be performed at low temperatures if exposed to light to avoid degradation.

Another aspect that could have influence on the storage of samples is the vial head space. Although no difference was observed in the concentration of tested isoflavones in HPLC vials with head space of 0–50% maintained at 10 and 25 °C for 7 days inside the climatic chamber, small differences were observed in the degradation of malonyl isoflavones at 40 °C. Malonyl isoflavones concentration (μM) of extracts inside vials with 50% of head space maintained at 40 °C were, in average, 6.60% lower than in extracts maintained vials with 0% head space. Degradation of isoflavone aglucones does not seems to be seriously affected by the head space of the HPLC vials, at least for short periods of time at low temperature (1–7 days). Therefore, this factor produces low effects on individual isoflavone concentration.

4. Conclusions

Although, degradation of isoflavones is affected by storage duration, temperature, light and by the vial head space, the importance of temperature cannot be overstated. It is the single most important parameter responsible for degradation of isoflavones. Increasing the storage duration and temperature resulted in severe changes in the isoflavone profile in the sample, mainly due to degradation of malonyl isoflavones to its respective glucosidic form. The most susceptible to degradation isoflavones are the malonyl forms and some aglucones are sensible to UV–Vis light. The exposure of the sample to UV–Vis light can be an important source of errors that should not be overlooked. Vial head space seems to have little effect on degradation of isoflavones. Isoflavone stored at temperatures between −20 and 10 °C retains individual original concentration even if stored with direct incident UV–Vis light for a week. Therefore, isoflavones must be kept at temperatures lower than 10 °C and protected from light to avoid degradation. Using these conditions, the extracts can be stored up to one week with no significant degradation of isoflavones allowing better planning of routine analysis of large number of samples, since the HPLC analysis can be performed several days after the extraction with accuracy and precision.

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