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# Optimization of acid hydrolysis conditions for total isoflavones analysis in soybean hypocotyls by using RSM

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**Abstract**

The optimum hydrolysis conditions of soybean hypocotyls with HCl for a quantitative HPLC determination of total isoflavones (TI) were studied by using response surface methodology (RSM). HCl concentration (HC), hydrolysis time (HT) and reaction temperature (RT) were assumed to be the most important factors affecting hydrolysis for the determination of TI. Optimum hydrolysis conditions for maximizing the determination of TI were: HC = 3.42N, HT = 205.5 min and RT = 44.6°C. The model had a satisfactory coefficient of $R^2$ (= 0.967) and was verified experimentally. Furthermore, the recovery of individual isoflavones was 97–100% under the optimum hydrolysis conditions. The results suggested that the conditions were mild and useful for maximizing a quantitative HPLC determination of TI in soybean hypocotyls. 

*Keywords:* Isoflavones; Acid hydrolysis; Optimization; Response surface methodology; Soybean hypocotyls

## 1. Introduction

Isoflavones are a class of flavonoids present in the human diet, mainly derived from soybean-based foods. The major dietary isoflavones, daidzein and genistein, have estrogen-like activity and are classified as phytoestrogens (Hodgson, Puddey, Berilin, Mori & Croft, 1998). Because the presence of isoflavones in human urine was related to lower mortality from sex hormone-dependent cancers, isoflavones have excited scientific researchers (Barnes & Messina, 1991; Zhang, Wang, Murphy & Hendrich, 1999). Isoflavones might be partly responsible for the ability of soybeans to lower the risk of cardiovascular diseases and to prevent bone mineral loss in ovariectomized rats (Anderson, Johnstone & Cook-Newell, 1995; Arjmandi et al., 1996). Daidzein, genistein and their corresponding glycosides account for the major portion of soy isoflavones and have been the focus of numerous studies.

Quantitative measurement of individual isoflavone glycosides in foods is difficult because most reference compounds are not commercially available. For example, 6''-*O*-malonylgenistin and 6″-*O*-malonyldaidzin are the major isoflavones of soybean hypocotyl and cotyledon as well as 6″-*O*-acetylgenistin and 6″-*O*-actyldaidzin are the major isoflavones of texturized vegetable protein (Kudou et al., 1991; Kurzer & Xu, 1997; Wang & Murphy, 1994). All these isoflavones are not commercially available. Furthermore, identification and quantification of each isoflavone requires tedious work and expensive equipment, such as mass spectrometry and nuclear magnetic resonance spectra (Kudou et al., 1991; Barnes, Coward, Kirk & Sfakianos, 1998). Therefore, hydrolysis of all glycoside derivatives to aglycones (daidzein and genistein) and/or β-glucoside conjugates (daidzin and genistin) was a practical method for the quantitative determination of total isoflavones in foods. Hydrolysis of isoflavones with HCl has been described by Wang et al. (1990). They concluded that soybeans and their processed products pretreated by 1N HCl for 2 h at 98–100°C were the best conditions for the hydrolysis of daidzein and genistein conjugates. However, acid hydrolysis at high temperature resulted in a notable degradation of genistein.

The objective of our investigation was to establish an acid hydrolysis method for a quantitative HPLC determination of total isoflavones (TI) including daidzin, genistin, daidzein and genistein in soybeans and their related products. The hypocotyl of soybeans was selected

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as representative due to its abundance in isoflavones. Response surface methodology (RSM) was employed to optimize the hydrolysis conditions, which could maximize the determination of TI in the hypocotyls.

## 2. Materials and methods

### 2.1. Materials

Soybeans were purchased from a local food store. The hypocotyls were obtained from soybeans by hand. Methanol, acetonitrile, trifluoroacetic acid (TFA), and HCl were purchased from Merck Co. (Darmstadt, Germany). Butylated hydroxytoluene (BHT) and isoflavone standards, daidzein, genistin and genistein, were purchased from Sigma Chemical Co. (St. Louis, MO). Daidzin was from Indofine Chemical Company, Inc. (Belle Mead, NJ).

### 2.2. Experimental design

The hypocotyls of soybean were heated at 90°C for 6 h and ground with a blender, then preserved in vacuum until use. Portions of ground hypocotyls (0.5 g) were mixed with 25 ml of methanol and the total volume was brought to 50 ml with HCl and/or distilled water according to the conditions described below. At the same time, 0.01% BHT (w/v) was added to each sample to prevent oxidation. Factors considered likely to affect acid hydrolysis were HCl concentration (HC = 0–6.0 N), hydrolysis time (HT = 0–350 min) and reaction temperature (RT = 5–95°C). Response surface methodology (RSM) (Cochran & Cox, 1957) was used to optimize the hydrolysis reaction parameters. A five-level three-factor fractional design (Box & Behnken, 1960) was adopted to optimize the hydrolysis conditions for analysis of total isoflavones in terms of daidzin, genistin, daidzein and genistein. The quadratic response surface model fitted Eq. (1):

$$Y = b_o + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 \quad (1)$$

where $Y$ is a response variable of total isoflavones. The $b_i$ are regression coefficients for linear effects; $b_{ik}$ are regression coefficients for effects from interaction; $b_{ii}$ are regression coefficients for quadratic effects; and $X_i$ are coded experimental levels of the variables. Statistical Analysis System (SAS Institute, Inc., 1988) was used to fit the second order polynomial equation to the experimental data.

### 2.3. Analysis of total isoflavones

HPLC analysis of total isoflavones was carried out on an Aquapore $C_8$ (250×4.6 mm i.d.; 7 μm) reversed-phase column (Waters, Milford, MA) coupled with Hitachi HPLC system (Model 7250, Tokyo, Japan) and a Hitachi UV detector (Model L-7400) according to Barnes, Kirk and Coward (1994). Elution was performed at a flow rate of 1.5 ml/min with the following solvent system: $A$ = 0.1% TFA (v/v), $B$ = acetonitrile; 100% $A$ was held for 10 min, followed by a linear decrease of $A$ from 100 to 50% in 20 min, and then subsequently decreased to 0% in 5 min by gradual addition of $B$. Eluted isoflavones were detected by their absorbance at 262 nm. Quantification of daidzin, genistin, daidzein and genistein was performed by comparison to known standards. All acid hydrolysates of samples were filtered with a Millipore Millex-HV 13 filter (0.45 μm) prior to injection.

### 2.4. Verification of model

Optimizations of hydrolysis conditions, including reaction temperature (RT), hydrolysis time (HT) and HCl concentration (HC) for maximizing a quantitative HPLC determination of total isoflavones (TI) in the hypocotyls were calculated by using the predictive equation from RSM. The actual determination of TI was carried out by HPLC after hydrolysis at the optimum conditions, and the result was compared to the predicted value.

### 2.5. Recovery of individual isoflavones

To test the recovery of isoflavones under the predicted optimum conditions, fixed amounts of individual daidzin, genistin, daidzein and genistein standards were separately used to conduct the experiment. The total amount of individual isoflavone standards after hydrolysis was determined by HPLC.

## 3. Results and discussion

The experiment was conducted by using a five-level three-factor central composite design with six replicates at the central point. The coded and actual levels of the three variables in Table 1 were selected to maximize the HPLC determination of total isoflavones (TI). Table 2 showed the treatments with coded levels and their

Table 1
Coded and actual levels of three variables

| | Coded level of variable | | | | |
|---|---|---|---|---|---|
| Variable | −1.68 | −1 | 0 | 1 | 1.68 |
| HCl concentration (HC, normality) | 0 | 1.22 | 3.00 | 4.78 | 6.00 |
| Hydrolysis time (HT, min) | 10.0 | 78.9 | 180.0 | 281.1 | 350.0 |
| Reaction temperature (RT, °C) | 5.0 | 23.3 | 50.0 | 76.8 | 95.0 |

experimental results of TI in the hypocotyls of soybean. Table 3 illustrates the coefficients of the regression model for the determination of TI. The results indicate that TI depended on the linear terms of HC ($P < 0.005$) and HT ($P < 0.05$), the quadratic terms of three variables ($P < 0.005$) and interactions of HC and HT as well as HT and RT ($P < 0.005$). The regression analysis showed that 96.7% of the variations was explained by the model.

The contour and three-dimensional plots presented in Figs. 1–3 were produced for each pair of factors, whereas the third factor was taken as a constant at its middle level. Fig. 1 shows the effects of HC and HT on the determination of TI in the hypocotyls of soybean. The maximum TI could be obtained with both HC and HT locating in the medium levels. Both Higher HC and extended HT resulted in the decrease of TI, which could

Table 2
Coded level combinations for a three-variable central composite orthogonal and rotatable design (CCD)

| | Coded level of variable[b] | | | |
|---|---|---|---|---|
| Test run[a] no. | HC (molarity) | HT (min) | RT (°C) | TI[c] (mg/g) |
| 1 | −1 | −1 | −1 | 6.3 |
| 2 | −1 | −1 | 1 | 10.21 |
| 3 | −1 | 1 | −1 | 8.19 |
| 4 | −1 | 1 | 1 | 9.09 |
| 5 | 1 | −1 | −1 | 9.86 |
| 6 | 1 | −1 | 1 | 8.19 |
| 7 | 1 | 1 | −1 | 11.6 |
| 8 | 1 | 1 | 1 | 7.70 |
| 9 | 0 | 0 | −1.68 | 7.68 |
| 10 | 0 | 0 | 1.68 | 7.38 |
| 11 | 0 | −1.68 | 0 | 8.96 |
| 12 | 0 | 1.68 | 0 | 9.98 |
| 13 | −1.68 | 0 | 0 | 7.14 |
| 14 | 1.68 | 0 | 0 | 8.68 |
| 15 | 0 | 0 | 0 | 11.7 |
| 16 | 0 | 0 | 0 | 11.53 |
| 17 | 0 | 0 | 0 | 11.7 |
| 18 | 0 | 0 | 0 | 11.8 |
| 19 | 0 | 0 | 0 | 11.7 |
| 20 | 0 | 0 | 0 | 11.7 |

[a] Test runs were performed in a random order.
[b] HC, HC1 concentration; HT, hydrolysis time; RT, reaction temperature.
[c] TI, total isoflavones.

Table 3
Coefficients of the quadratic regression model for the determination of total isoflavones in the hypocotyls of soybean

| Effect[a] | Regression coefficient | *T* test |
|---|---|---|
| *Intercept* | b0 = 11.66 | 61.77** |
| X1 = HC | b1 = 0.45 | 3.602** |
| X2 = HT | b2 = 0.27 | 2.192* |
| X3 = RT | b3 = −0.09 | −0.735 |
| *Quadratic* | | |
| X11 | b11 = −1.17 | −9.57** |
| X22 | b22 = −0.61 | −4.02** |
| X33 | b33 = −1.30 | −10.68** |
| *Interaction* | | |
| X1×X2 | b12 = −0.06 | 0.373 |
| X1×X3 | b13 = −1.30 | −7.94** |
| X2×X3 | b23 = −0.66 | −4.02** |
| $R^2 = 0.967$ | | |

[a] HC, HC1 concentration; HT, hydrolysis time; RT, reaction temperature; TI, total isoflavones.
*$P < 0.05$.
**$P < 0.005$.

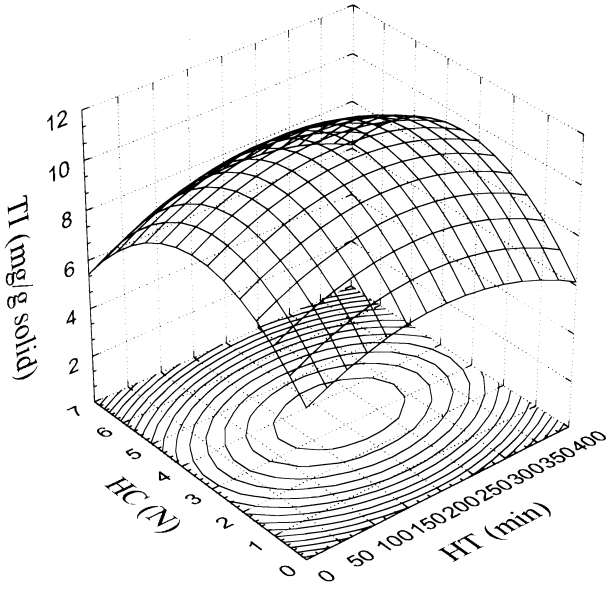


Fig. 1. Effects of HCl concentration (HC) and hydrolysis time (HT) at 50°C on the determination of total isoflavones (TI) in the hypocotyls of soybean.

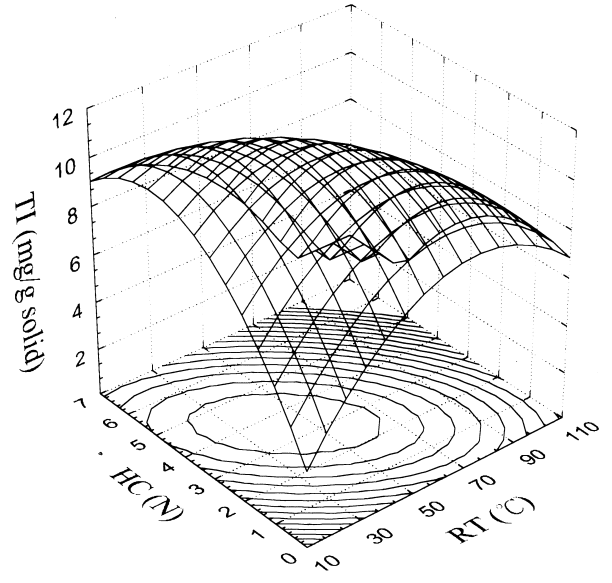


Fig. 2. Effects of HCl concentration (HC) and reaction temperature (RT) in 180 min hydrolysis on the determination of total isoflavones (TI) in the hypocotyls of soybean.

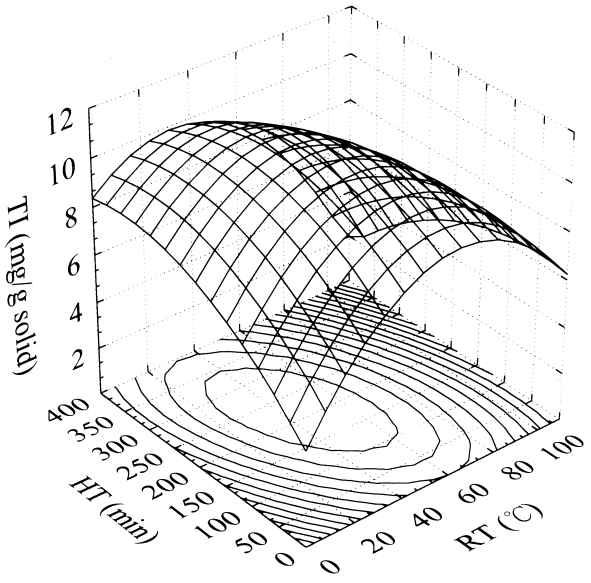


Fig. 3. Effects of hydrolysis time (HT) and reaction temperature (RT) in hydrolysis with 3N HCl on the determination of total isoflavones (TI) in the hypocotyls of soybean.

be due to the degradation of genistein. Wang et al. (1990) studied the effect of HC and HT on daidzein and genistein concentration at 99–100°C, individually. They concluded that daidzein concentration increased with HT, reached a maximum and then levelled off, while genistein concentration tended to decrease after reaching the maximum due to the degradation of the molecule by HCl. Lower HC and shorter HT, however, might result in only partial hydrolysis of conjugated isoflvones, which would result in underestimation of TI.

Fig. 2 illustrates the effects of HC and RT on the determination of TI in the hypocotyls of soybean. The maximum TI was obtained with HC locating between 3 and 4N and RT locating between 30 and 50°C. Higher HC and longer RT tended to result in a decrease of TI. This also could be due to the degradation of genistein. Wang et al. (1990) studied the hydrolysis of conjugated daidzein and genistein. Their study indicated that genistein concentration would notably decrease at an HC higher than 2N at 99–100°C. Fig. 3 shows the effects of HT and RT on the determination of TI. The maximum TI was obtained with HT locating between 150 and 300 min and RT locating between 40 and 60°C.

Analysis of the surface response revealed that the stationary point for the determination of TI in soybean hypocotyls was a true maximum. The maximum predicted value of TI was 11.82 mg/g hypocotyl under the following hydrolysis conditions: HC at 3.42N, HT at 205.5 min and RT at 44.6°C. Based on the regression analysis, the model had a satisfactory coefficient of $R^2 = 0.967$. Furthermore, the verification studies also proved that the predicted value of TI for the model could be realistically achieved within a 95% confidence interval of experimental values (Table 4).

Table 4
Results of verification tests for the fitted models

| Response variable | TI[a] (mg/g hypocotyl) |
|---|---|
| Predicted value | 11.8 |
| Experimental value (mean) | 11.9 |
| Sample size | 7 |
| 95% Confidence interval | (11.7, 12.0) |

[a] TI, total isoflavones.

To examine the possible degradation of isoflavones under the acid hydrolysis conditions of 3.42N HCl, 205.5 min and 44.6°C, the recoveries of daidzein, genistein, daidzin and genistin were inspected, individually. The results indicated that the recoveries of genistein approached 97% and all the others, daidzein, daidzin and genistin, reached 100%. The efficiency of extraction TI from the hypocotyls was also examined by hydrolysis with 3.42N HCl at 44.6°C within 30–360 min. The results indicated that TI increased when HT increased from 30 to 180 min, and then levelled off from 180 to 210 min. As HT was longer than 210 min, TI tended to decrease (data not shown). Therefore, the optimun HT of 205.5 min was proved to be enough to extract TI completely from the hypocotyls. The above results suggested that the acid hydrolysis conditions were mild and useful for maximizing a quantitative HPLC determination of TI in the hypocotyls of soybean.

## 4. Conclusion

The high correlation of the model showed that second order polynomials could be used to optimize the hydrolysis conditions for maximizing the HPLC determination of total isoflavones in the hypocotyls of soybean. Optimum conditions for acid hydrolysis were: HCl concentration, 3.42N; hydrolysis time, 205.5 min; and reaction temperature, 44.6°C. The conditions allow a fast, quantitative and maximum determination of total isoflavones, including daidzein genistein, daidzin and genistin in the hypocotyls of soybean. As a large number of different glucosides are present in food, the quantitative determination of individual isoflavone glucosides in soy products would be complicated. Therefore, analysis of aglycones and β-glucoside conjugates after acid hydrolysis will be a practical method for the quantitative determination of total isoflavones in foods.

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## References


Anderson, J. M., Johnstone, B. M., & Cook-Newell, M. E. (1995). Meta-analysis of the effects of soy protein intake on serum lipids. *The New England Jouranl of Medicine, 333*, 276–282.

Arjmandi, B. H., Alekel, L., Hollis, B. W., Amin, D., Stacewicz-Aapuntzakis, M., Guo, P., & Kukreja, S. C. (1996). Dietary soybean protein prevents bone loss in an ovariectomized rat model of osteoporosis. *Journal of Nutrition, 126*, 161–167.

Barnes, S., & Messina, M. (1991). The role of soy products in reducing cancer risk. *Journal of the National Cancer Institute, 83*, 541–546.

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*, 2466–2474.

Barnes, S., Coward, L., Kirk, M., & Sfakianos, J. (1998). HPLC-mass spectrometry analysis of isoflavones. *Proceedings of the Society for Experimental Biology and Medicine, 217*, 254–262.

Box, G. E. P., & Behnken, D. W. (1960). Some new three level designs for the study of quantitative variables. *Technometrics, 2*, 455–475.

Cochran, W. G., & Cox, G. M. (1957). *Experimental design* (2nd ed.) (pp. 335–375). New York: John Wiley & Sons, Inc.

Hodgson, J. M., Puddey, I. B., Beilin, L. J., Mori, T. A., & Croft, K. D. (1998). Supplementation with isoflavonoid phytoestrogens does not alter serum lipid concentrations: a randomized controlled trial in humans. *Journal of Nutrition, 128*, 728–732.

Kudou, S., Fleury, Y., Welti, D., Magnolato, D., Uchida, T., Kitamura, K., & Okubo, K. (1991). Malonyl isoflavone glycosides in soybean seeds (Glycine max MERRILL). *Agricultural and Biological Chemistry, 55*, 2227–2233.

Kurzer, M. S., & Xu, X. (1997). Dietary phytoestrogen. *Annual Review of Nutrition, 17*, 353–381.

SAS Institute, Inc. (1988). *SAS/STAT TM user's guide, release 6.03 edition*. Cary, NC: Author.

Wang, G., Kuan, S. S., Francis, O. J., Ware, G. M., & Carman, A. S. (1990). A simplified HPLC method for the determination of phytoestrogens in soybean and its processed products. *Journal of Agricultural and Food Chemistry, 38*, 185–190.

Wang, H. J., & Murphy, P. A. (1994). Isoflavone composition of American and Japanese soybeans in Iowa: effects of variety, crop year, and location. *Journal of Agricultural and Food Chemistry, 42*, 1674–1677.

Zhang, Y., Wang, T. T. S., Murphy, P. A., & Hendrich, S. (1999). Urinary disposition of the soybean isoflavones daidzein, genistein and glycitein differs among humans with moderate fecal isoflavone degradation activity. *Journal of Nutrition, 129*, 957–962.