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QUANTIFICATION OF SOY ISOFLAVONES IN MEAT PRODUCTS BY HPLC

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Abstract

A fast, specific, and sensitive method for determination of soy additives in processed meat products has been developed. The method is based on RP-HPLC analysis of the isoflavones daidzein and genistein using bisphenol A as an internal standard. This paper describes the development and optimisation of efficient extraction procedures (n-hexane/80% EtOH), concentration of isoflavones by solid-phase extraction (Spe-ed ABN cartridges), and conditions for gradient RP-HPLC analysis. The presence of soy additives in different types of sausages was determined by this method. As little as 0.1 % of soy additives in heated meat products could be detected and the quantitative determination of soy proteins is possible in the range of 0.1–10 %.

Key words

Meat products, Soy proteins, Isoflavones, Daidzein, Genistein

INTRODUCTION

Soy proteins in the form of concentrates, isolates and texturised products are now used in a large variety of meat products. Soy proteins are added to meat products to enhance the emulsifying and water-binding capacity of meat proteins. Because of regulations allowing only certain quantities of soy proteins in foodstuffs, their detection is currently of great significance. The most widespread markers that can be used for qualitative and quantitative determination of additives in food products are nucleic acids (*1,2*) and proteins (*3–5*). On the other hand, plant additives contain a large amount of low-molecular weight substances, which can serve as suitable analytical markers. Daidzein and genistein are the major isoflavones found in soy protein additives (*8,9*). A number of methods, including immunological (ELISA), electrophoretic and chromatographic methods, have been applied to determine the amount of soy additives in foodstuffs (*10–13*). The reversed phase (RP), by far the mostly used mode of high performance liquid chromatography (HPLC), is a method widely used for quantitative determination of daidzein and genistein (*10, 14–17*).

The aim of this work is to optimise the procedures of extraction of isoflavones from meat products, to test the possible methods of concentration of isoflavones in analysed samples, and to develop a relevant HPLC method for determination of soy additives by using isoflavones as markers of presence of soy additives in meat products.

MATERIAL AND METHODS

Chemicals and reagents. Daidzein, genistein, flavone, bisphenol A, and butylated hydroxytoluene (BHT) were supplied by Sigma-Aldrich (Germany). Acetonitrile, ethanol, methanol, acetone, and n-hexane (HPLC grade) are Riedel-de Haän products. Acetic acid and other chemicals (analytical grade) were purchased from Sigma-Aldrich (Germany). Samples of beef and pork meat were purchased from local supermarkets, the other meat products as well as soy additives were received from a local manufacturer (soy isolate "SUPRO" content of soy proteins 90 % and soy concentrate "DANPRO" content of soy proteins 70 %). Stock standard solutions of daidzein, genistein, and bisphenol A were prepared by dissolving standards in DMSO. Approximately 15 mg each of daidzein, genistein, bisphenol A, and flavone were accurately weighed and dissolved in 1.5 ml DMSO. The concentration of the stock solutions was determined by absorbance measurement at the wavelength with maximum absorption (λmax) using the following values of molar extinction coefficients (ε): genistein, λmax = 263 nm, ε = 37 154 $l.mol^{-1}.cm^{-1}$; daidzein, λmax =250 nm, ε = 20 893 $l.mol^{-1}.cm^{-1}$ (*7*). The stock solutions were stored at -20 °C prior to use.

Equipment. The HPLC system, assembled from SHIMADZU modular components, consisting of a model LC-10AD pump, a model SPD-M10AVP diode array detector, and an interface module was used for this analysis. The samples (20 μl) were injected into a SUPELCOSIL LC-18-DB column (250 x 4.6 mm, i.d. 5 μm). Elution was carried out at a flow rate of 1 ml/min. The following mobile phase and gradient programme were used: mobile phase A: 10 % acetic acid, mobile phase B: 100 % acetonitrile. Gradient: linear gradient: from 35 to 100 % B over 10 min, isocratic at 100 % B over 10 min, and isocratic at 35 % B for 20 min. Spe-ed ABN cartridges were obtained from Applied Separation, Allentown (USA).

Extraction procedures. Extraction of fat: before extraction of fat the model mixtures of meats (beef : pork, 1: 2) and soy isolates (0.5–10 %) (Supro or Danpro) were heated for 10–60 min at 70 °C. The sausages or model meat products were ground three times to obtain a homogenised mixture. Ten grams of each sample were mixed with 50 ml of n-hexane, thoroughly vortex-mixed for 20 min. This procedure was repeated three times. The sample was centrifuged for 10 min at 8500 g. Defatted meat samples were dried and isoflavones were extracted from these matrices. The internal standard bisphenol A or flavone (60 μl DMSO solution) and BHT as antioxidant (0.05 %) were added to all samples at the beginning of the extraction procedure.

Extraction of isoflavones: The extraction of daidzein and genistein from dry foodstuff powder was performed by 15 min sonication in 20 ml of 80% ethanol. After centrifugation the ethanolic solution was transferred into disposable tubes and ethanol was evaporated and the isoflavones were finally dissolved in 2 ml of ethanol.

Solid-phase extraction: In the case of samples with a lower isoflavone content (sausages) a solid-phase extraction (SPE) with Spe-ed ABN cartridges was carried out for trace enrichment of isoflavones (*14*). The cartridges were first equilibrated with 3 ml of 99% methanol and consequently with 3 ml of water. Six ml of each sample (2 ml of ethanolic solution obtained within step II was mixed with 4 ml of distilled water) was passed through the cartridge. The impurities were washed out with 3 ml of 5% methanol and isoflavones were eluted with 3 ml of 80% methanol. The samples were then evaporated and the dry residues were finally dissolved in 100 μl of 80% ethanol and analysed by HPLC.

Identification of isoflavones: Both daidzein and genistein extracted from meat products and from model mixtures were analysed by HPLC and identified by comparing retention times and UV absorption

spectra with the standards analysed by the same procedure (*Table 1*). Validation procedures. The calibration curves were obtained for each standard by expressing the peak area obtained from HPLC analyses with 20 μl injection as a function of standard concentration (7–9 samples).

Table 1
HPLC and calibration parameters of isoflavones and internal standard

Compound	Retention time (min)	Range of linearity of calibration curve (μmol/l)	Mean recoveries (n= 3) (%)
Daidzein	5.5	5–50	97± 2
Genistein	7.7	25–180	99± 2
Bisphenol A	10.3	–	101± 3

The highest linearity and sensitivity of determination was obtained in the case of genistein, which was used as a marker for determination of soy protein in meat products. The precision of the method (R.S.D.) was calculated from seven injections of food samples containing known amounts of genistein. Extraction recovery was measured by the addition of a standard solution of daidzein and genistein into the food samples at a final concentration of 10 μg/ml. The spiked samples were then processed through the whole procedure.

Table 2
Total daidzein and genistein levels in soy additives obtained by proposed method

Sample	Daidzein and genistein in soy additives (mg /kg)	
	Daidzein	Genistein
Soy isolates	10.85 ± 0.36	15.12 ± 0.53
Soy concentrates	1.15 ± 0,12	1.49 ± 0.18

Table 3
The amount of soy additives in analysed meat products

Sample	Per cent of soy additives
Sausages	0.99 ± 0.22
Fine sausages	0.98 ± 0.30
Chicken sausages	1.05 ± 0.06
High-fat sausages	3.26 ± 0.62
Sausages with cheese	5.13 ± 0.42
Ham select quality	0
“Pizza” ham	1.53 ± 0.32
“Junior” salami	4.53 ± 0.12
“Vysočina” salami	0
Meat sausage	0.96 ± 0.29
“Ostravská” meat sausage	0

RESULTS AND DISCUSSION

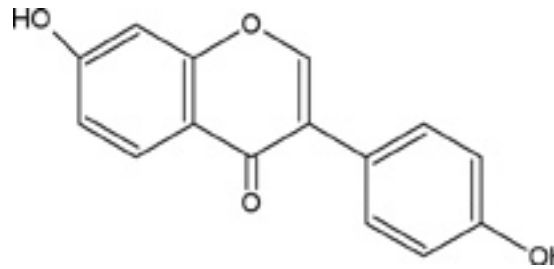
Soy protein additives contain isoflavones, i.e. daidzein and genistein are the major components of isoflavones. Their amount is sufficient for determination of soy proteins in processed soy products. In order to determine daidzein and genistein, which are present in processed meat foods, the samples were defatted, hydrolysed, and isoflavones were concentrated by using solid-phase extraction. Finally the amount of isoflavones was determined by HPLC with gradient elution (*Table 3*).

Thermal stability: The stability of analysed isoflavones at a higher temperature was tested by heating the model samples to 70 °C for different time periods (10–30 min). From the results it is clear (data not shown) that thermal treatment did not cause any decomposing effects; no influence on HPLC performance was observed.

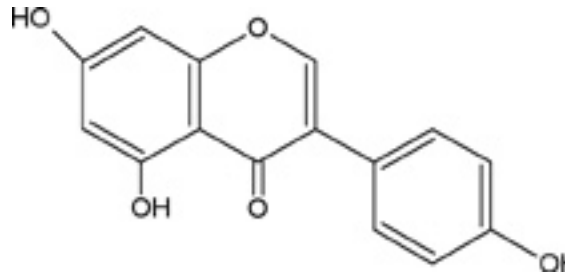
Defatting procedure: It was found that the presence of fat in model mixtures and real food samples at a level up to 20 % does not show any influence on extraction of isoflavones. However, the recovery and reproducibility of SPE extraction were strongly influenced at higher levels of fat (20–40 %), which is typical of most meat products (sausages) analysed by our procedure. We tested two defatting procedures with acetone and n-hexane. The defatting procedure using n-hexane resulted in the highest and more reproducible results for both analysed isoflavones.

Extraction of isoflavones: Different hydrolytic methods have been used for isoflavone extractions; most of them use refluxing with hydrochloric acid in the presence of ethanol. On the other hand, there are some reports indicating genistein to be unstable under acid hydrolysis conditions (*7*). Because of this we used an 80% ethanol/water mixture without acidification in connection with solid-phase extraction on Spe-ed ABN cartridges for the extraction of isoflavones. SPE was found as a very useful procedure; the recoveries were greater than 90 % in all cases.

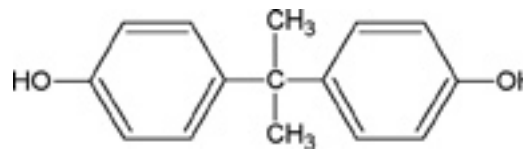
HPLC analysis: At first, isocratic elution of isoflavones with acetonitrile (60%)-acetic acid (4%) as a mobile phase was chosen. This procedure resulted in a relatively poor selectivity and peak shape for both daidzein and genistein (data not shown). The necessary time of elution was more than 45 min. For these reasons we tested different buffer systems and gradient conditions; finally acetic acid/acetonitrile appeared to afford the best peak resolution and shape. The analyte peaks and the peak of internal standard bisphenol A were well separated from interference of components of the matrices. At first we tested flavone as an internal standard. The position of its peak on HPLC chromatogram (R_t = 15 min) interfered with the position of peaks of matrices. The analytes were identified by comparing retention times and their UV absorption patterns with standards analysed under the same conditions. Representative chromatograms of some analysed samples are shown in *Fig. 2*. Total daidzein and genistein levels in soy additives obtained by the method proposed are given in *Table 2*.



DAIDZEIN



GENISTEIN



BISPHENOL A

Fig. 1
Structures of isoflavones and internal standard

SPE coupled with HPLC was successfully applied to the analysis of soy in processed meat products. According to the results, the separation of genistein could be achieved within 15 min. The equation of the calibration curve (genistein) follows: $y = 0.72 \cdot 10^6.x$. The sensitivity and range of linearity of the calibration curve have proved adequate for most determinations to be made (*Table 1*). The amount of soy additives in the meat products analysed was assessed on the basis of the genistein content in the model samples containing 0–10% soy isolate. The limit of detection is below 0.1 % of soy additives in meat products and quantitative determination of soy additives is possible in the range of 0.1–10 %. The heat processing applied to the meat products does not influence the applicability of the method developed.

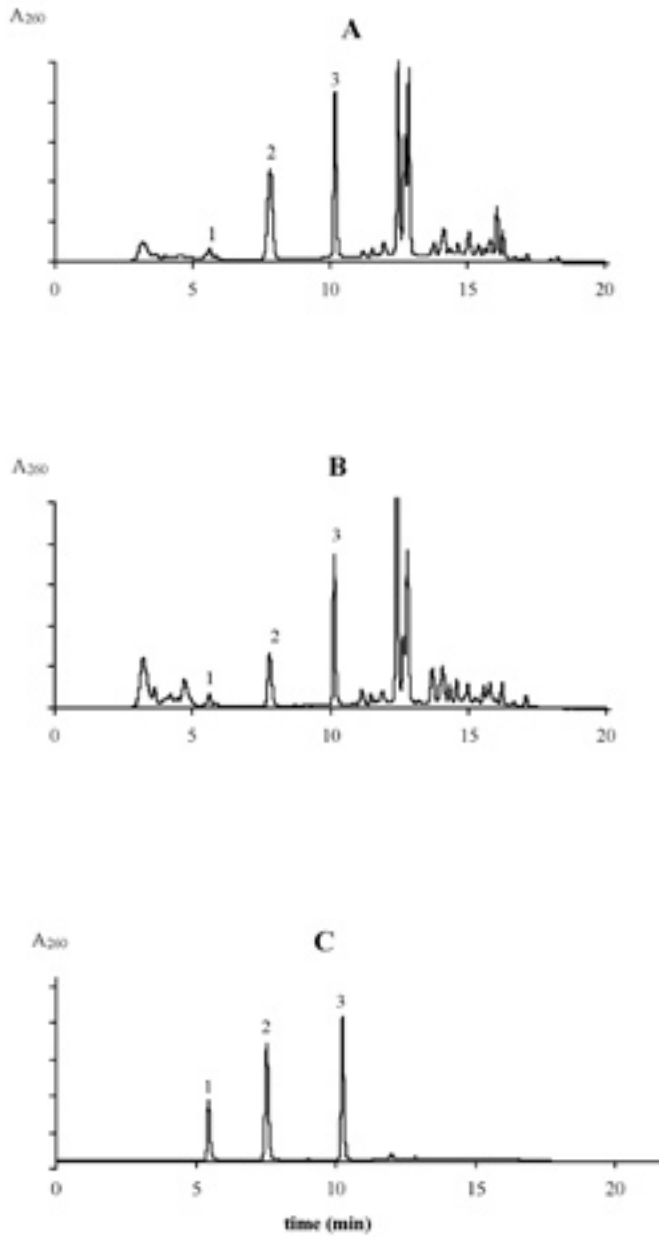


Fig. 2
HPLC- chromatograms of extract from chicken sausages (A), extract from model mixtures of meats (beef:pork, 1:2) + 1 % of isoflavones and bisphenol A (B), and standard solutions of daidzein (1), genistein (1), and bisphenol A (3) (C).

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KVANTIFIKACE IZOFLAVONŮ DAIDZEINU A GENISTEINU V MASNÝCH PRODUKTECH POMOCÍ HPLC

Souhrn

Byla navržena rychlá, specifická a citlivá metoda ke stanovení sójových aditiv v tepelně opracovaných masných výrobcích. Metoda je založena na RP-HPLC analýze izoflavonů daidzeinu a genisteinu a bisfenolu A jako vnitřního standardu. Toto sdělení popisuje návrh a optimalizaci efektivní extrakční procedury (n-hexan / 80% EtOH) a následné zakoncentrování izoflavonů metodou extrakce v pevné fázi (Spe-ed ABN kolonky) a podmínky gradientové RP-HPLC analýzy. Navržená metoda umožnila stanovit přítomnost sójových aditiv v různých typech párků. V tepelně opracovaných masných výrobcích je možno navrženým postupem detekovat přítomnost méně než 0,1 % sójových aditiv a jejich kvantitativní stanovení je možné v rozsahu 0,1–10 %.

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