Analysis of Polyphenols Using Capillary Zone Electrophoresis and HPLC: Detection of Soy, Lupin, and Pea Protein in Meat Products

O. Mellenthin and R. Galensa*

Institut für Lebensmittelwissenschaft und Lebensmittelchemie, Universität Bonn, Endenicher Allee 11-13, D-53115 Bonn, Germany

The analysis of polyphenols, which are characteristic of certain legumes, enables a rapid and sensitive detection of legume proteins in meat products. Separation of specific isoflavones can be achieved by capillary zone electrophoresis (CZE) or high-performance liquid chromatography (HPLC), both coupled with a photodiode array detector (DAD). The use of CZE in the identification process is an appropriate means of rapid screening; the HPLC is less dependent on matrix effects and clearly more sensitive. Additives of soy protein isolates up to 0.1% could be detected in meat products, even in sausages heated to a high temperature or with hydrolyzed soy proteins. A solid-phase extraction procedure with polyamide cartridges has been developed to concentrate polyphenols. A similar detection of lupin protein is possible in principle. In the case of pea protein, a reliable detection was not possible depending on the coincidental appearance of polyphenols as indicating substances.

Keywords: *Polyphenols; isoflavones; genistein; legume protein; soy; lupin; pea; meat products; capillary electrophoresis; CZE; HPLC; diode array detection; solid-phase extraction*

INTRODUCTION

This article deals with the investigation on the analysis of polyphenols in soy, lupin, and pea, using capillary zone electrophoresis (CZE) and HPLC, for the detection of these legume proteins in meat products.

The addition of a small amount of soy, lupin, or pea protein to processed meat products increases the shelf life of the products, the water retention, and the binding of fat by emulsification and gelation (Verma et al., 1984; McMindes, 1991; Lecomte et al., 1993; Pedersen, 1995; Alamanou et al., 1996). So the addition of these plant proteins to meat products can be very profitable. Manufacturers are increasingly using plant proteins in this way. Usually the content is 1−6% by weight of the final product (Gnanasambandam and Zayas, 1992; Ho et al., 1997). Important examples of processed foodstuff that contain legume proteins are comminuted products such as emulsified meats (frankfurters), liver sausages, and coarse-ground meats (ground beef patties, meatballs, pizza toppings) (Sipos, 1982; Lecomte et al., 1993; Pedersen, 1995; Ho et al., 1997). In addition soy protein is used in bakery products for its water adsorption and tenderizing effects, to provide body and resilience, but also to increase the shelf life of the products (Sipos, 1982).

Recently the importation of genetically modified soy beans, mixed with unmodified beans, has been reported. There is consumer concern in the EU about the use of such products. Consequently some food manufacturers have reacted by starting to avoid the use of soy as an additive. From this political environment the detection of soy within all kinds of foodstuff has become an important scientific issue. Currently there is a growing industrial interest in exploiting other indigenous vegetable protein sources such as lupin and pea, instead of soy. Lupin has a higher protein content than soy along with a similar amino acid profile. In this respect, lupin has been called “the new soy” (Lopez-Bellido and Fuentes, 1986). Other advantages of lupin are its lower habitat requirements which facilitates its cultivation in Northern Europe and the highlands of Africa and North and South America. Furthermore, lupin already plays an important role as a forage and as a green manure plant.

The use of plant proteins in meat products was prohibited by law in Germany until the end of 1995. Since then, their use requires labeling to protect the consumer from deception and guard against the hazards of serious, perhaps life threatening, allergic reactions (German Fleisch-Verordnung). Both soy and lupin proteins are known for their allergenic potential (Vieths et al., 1994; Hefle et al., 1994). Their detection in meat products is currently of great significance in Germany. Most of the methods used to detect soy protein are based on immunological protein differentiation (Brehmer, 1984). The enzyme-linked immunosorbent assay (ELISA) even gives quantitative results. The drawbacks of those methods are the high price and the risk of false negative results due to denaturation of the proteins during processing (Hitchcock et al., 1981; Strähle and Roth, 1996). Immunological methods may especially fail in the case of hydrolyzed samples or with food heated to a high temperature during manufacture.

The proposed method is based on the detection of naturally occurring polyphenols of the legumes. Polyphenols and isoflavones in particular are exclusively derived from the secondary metabolism of plants. Their occurrence in meat products can only be due to the use of spices or other vegetable ingredients. An important requirement for the proposed detection of soy and related legumes is the fact that naturally occurring isoflavones are found very specifically only within a few plant species (Fabaceae), such as soy, lupin, peanut,

* Corresponding author (fax 0049-228-733757; e-mail Galensa@uni-bonn.de).

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fenugreek, lentil, and pea. Moreover, the polyphenolic aglycons are largely insensitive to heat and alterations in the character of the proteins are unimportant (Mellenthin and Galensa, 1996). A complete separation of the polyphenols from the proteins is hard to achieve in the manufacturing process. Therefore, the method can be used as an alternative routine or reference method to immunological methods. It was initially developed and tested for the rapid detection of soy protein in meat products (Mellenthin and Galensa, 1996, 1997a,b). For that purpose standards of soy isoflavones as well as different soy products were measured, using CZE and high-performance liquid chromatography (HPLC), both coupled with a photodiode array detector.

EXPERIMENTAL PROCEDURES

Standards. Stock solutions of genistein (Sigma), genistin (Sigma), daidzein (ICN), and daidzin (Roth) were prepared in the concentration range of 200−400 mg/L using *N*,*N*-dimethylformamide (DMF)/water (2 + 1, v + v). They were diluted 1:100 with extraction solvent before the measurements. Standards of 2′-hydroxygenistein, wighteone, luteone, and lupinalbin A were received from Plantech (Reading, U.K.). The pisatin standard was obtained from the Department of Plant Biology, University of Maryland, College Park, MD.

Food Samples. Soy products as well as cakes and bread with soy contents were received from a soybean company. Sausages with known amounts of soy, lupin, and pea proteins (0.5−6%) were obtained from industrial manufacturers, including ham sausages (Rheinische Schinkenwurst), liver sausages, canned sausages, and salami. These companies also supplied different isolated soy, lupin, and pea proteins.

Numerous routine food control samples (meat products with soy protein contents of up to 0.1%, ready cooked meals, cheese, pastries) were received from the Bergisches Land Chemical Testing Laboratory, Germany (Wuppertal, Chemisches Untersuchungsinstitut Bergisches Land), and different industrial manufacturers.

Extraction and Cleanup Procedure. Sausages with firm texture like salami and ham sausages were deep-frozen using liquid nitrogen and crushed to powder for 3 min (amplitude 100%) using a ball-mill (Retsch MM 2000). Other sausages were homogenized and extracted without further treatment. The isoflavonoids were extracted from food matrixes (e.g. meat products) using *N*,*N*-dimethylformamide (DMF)/water (2+1, v+v) or the extraction solvent of Shihabi et al. (1994) (acetonitrile/water, 2+1, v+v).

For quantitative investigations using CZE an addition of 3-isobutyl-1-methylxanthin (Sigma) as an internal standard (ISTD) to the extraction solvent is recommended. An amount of about 2 g of a homogenized meat sample was extracted with 5 mL of the extraction solvent in an ultrasonic bath (Bandelin Sonorex RK100H) for 5 min. The extract was filtered and used for the CZE and HPLC determinations.

For samples with low isoflavone content or with a matrix interfering with the experiment, a solid-phase extraction (SPE) with polyamide (PA) or alternatively with RP18 cartridges (both from Macherey-Nagel) was carried out (Mellenthin and Galensa, 1997b). The cartridges contained 1000 mg of the solid phase. RP18 columns had to be conditioned with two column volumes of methanol followed by the same volume of water. The pretreatment of the polyamide column was achieved with two column volumes of water only. With both types of cartridges the applied samples had to be free of organic solvent. Polyamide as well as RP18 was washed with 20 mL of water to remove chromatographical interferences. Afterward the polyphenols were eluted with 20 mL of methanol in both cases. The eluate was concentrated using a rotary evaporator, and the residue was made up to volume with acetonitrile/water (2+2, v+v; e.g. 1−5 mL depending on the sample analyzed).

Apparatus. The CZE separations were performed using a P/ACE 5500 capillary electrophoresis system (Beckman Instruments, San Ramon, CA), equipped with a fused-silica capillary 57/50 cm or 85/78 cm, both of 50 or 75 μm i.d. The separation buffer was 200 mmol/L boric acid (adjusted to a pH of 8.6 with NaOH). The other conditions were the following: conditioning of the capillary, 3 min with NaOH (0.5 mol/L) and 4 min with the boric acid buffer before each run; sample injection, 5 s with pressure (1.38 bar); voltage, 25 or 30 kV; temperature, 25 °C; detection wavelength, 260 nm (soy) or 270 nm (lupin, pea); DAD scans, from 200 to 400 nm.

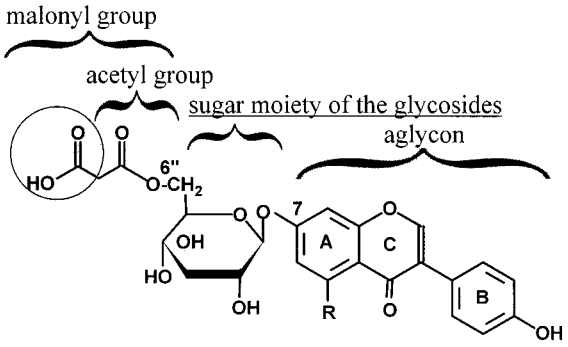

Figure 1. Chemical structures of soy isoflavones: R = H (daidzein and derivatives); R = OH (genistein and derivatives).

The liquid chromatograph consisted of a programmable solvent Module 125, a diode array detector Module 168, and a Triathlon autosampler 507 (all from Beckman Instruments, San Ramon, CA). The column was an ODS Ultrasphere 250 mm × 4.6 mm i.d., 5 μm (Beckman Instruments, San Ramon, CA). Flow rate was 0.9 mL/min, and the injection valve volume, 20 μL. Eluent A was phosphoric acid (0.1%) in water; eluent B, acetonitrile. Gradient profiles: determination of soy, linear gradient from 5% B to 35% B in 45 min, in 20 min to 100% B and then reset; determination of lupin and pea, linear gradient from 10% B to 35% B in 35 min, in 20 min to 65% B, 5 min isocratic elution, in 10 min to 100% B and then reset.

As in CZE the detection wavelength was set at 260 nm (soy) and 270 nm (lupin, pea). DAD spectra were recorded from 200 to 400 nm.

For the HPLC/MS-coupling experiments eluent A was acetic acid (2%) in water, eluent B acetonitrile, and gradient the same as above. The thermospray mass spectrometric measurements were performed using an SSQ 710 mass spectrometer with TSP-ion source and with an TSP-1 interface (Finnigan MAT, San Jose, CA). Different modes were used as follows. (1) Buffer mode: addition of 0.5 mL/min ammonium acetate buffer (0.1 mol/L, with addition of 10% methanol); vaporizer temperature, 90 °C; aerosol temperature, 240 °C; *m*/*z* range, 150−600. (2) Discharge mode: without buffer addition, discharge with 50 μA. The preliminary tests with an electrospray coupling were performed using a quadrupole ion trap mass spectrometer (LCQ), Finnigan MAT (San Jose, CA). The ESI energy was −5 kV, and helium was used as collision gas with energies of 16−21%.

RESULTS AND DISCUSSION

1. Detection of Soy Protein. *Significance of Target Compounds.* The aglycones genistein, daidzein, and their corresponding glycosides turned out to be suitable indicators for soy protein addition. They not only occurred in all soybean species but also in every isolated soy protein, soy protein concentrate, and the aglycones even in soy lecithin. Other major isoflavones in soy are the 6″-*O*-malonyl and 6″-*O*-acetyl derivatives (Wang and Murphy, 1994). Figure 1 gives the chemical structures of soy isoflavones.

Advantages of the Cleanup Procedure. Since isoflavones are readily soluble, even from complex matrixes such as meat products, they were extracted in 5 min using an extraction solvent consisting of acetonitrile and

water. The advantage of this solvent system was the low ability to extract fat which could interfere with the measurements by affecting the electroosmotic flow (EOF). For the rapid and semiquantitative determination (screening) of soy in food only one extraction step was necessary with a recovery of about 70% of the total extractable isoflavone content. For quantitative results three extraction steps, each followed by a centrifugation, were carried out. The filtered extract could be used directly for the CZE and HPLC determination.

For samples with low isoflavone contents or interfering matrix constituents a solid-phase extraction procedure (SPE) with RP18 or polyamide cartridges was necessary, which was more time-consuming than the direct extraction. With both solid phases it was possible to concentrate isoflavones. Polyamide was superior to RP18 as more specific interactions with polyphenols (via hydrogen bonds) occur. A minimum of interfering matrix peaks was eluted together with the isoflavones. Using this method it was possible to improve the detection limit (0.1−0.5% of isolated soy protein, using HPLC) by a factor of 10, in some cases—depending on the matrix—even by a factor of 50−100. Sometimes a combination of polyamide and RP18 can be used to yield optimal removal of matrix compounds interfering with the sample measurement.

Comparison between CZE and HPLC. Soy isoflavones can be separated with both HPLC and CZE in a single run. Since the separation mechanisms in CZE and HPLC are completely different, the analysis of a sample with both methods gives two independent proofs of the results. Figure 2 shows a comparison between the CZE and HPLC separation of soy isoflavones.

During CZE separation toward the cathode, the glycosides daidzin and genistin with their higher molecular weights were detected earlier than the aglycones. This is because the negatively charged isoflavone molecules (buffer pH of 8.6) move toward the anode and against the EOF. Borate as a complexing agent in the CZE buffer increased the separation, although genistein, daidzein, and glucose are not able to form stable complexes with borate because they are lacking orthodihydroxy groupings (aglycones) or vicinal hydroxy groups with cis configuration (sugar moiety). According to McGhie and Markham (1994), glycosylation of free phenolic groups results in a decrease in the electrophoretic mobilities due to an increase in molecular size and a reduction in molecular charge from loss of ionizable phenolic groups. The smaller aglycone molecules thus have a higher mobility toward the anode and against the EOF which transports them to the detector. The detection of daidzin before genistin and daidzein before genistein can be explained by charge effects (the extra hydroxy group of genistein which tends to lose a proton). Migration times of the four standards and standard addition to soy products as well as the UV spectra were used for the identification process. The reproducibility of migration times in the CZE runs was relatively poor, although the uncoated fused silica capillaries were rinsed with alkali and the borate buffer before each run. The main advantage of CZE is the short analysis time (8 min).

In contrast, the chromatographic separation using HPLC requires a period of 50 min and additional equilibration time. The elution order in the HPLC investigations was in tune with Wang and Murphy (1994): glucosides, 6″-*O*-malonyl glucosides, 6″-*O*-acetyl

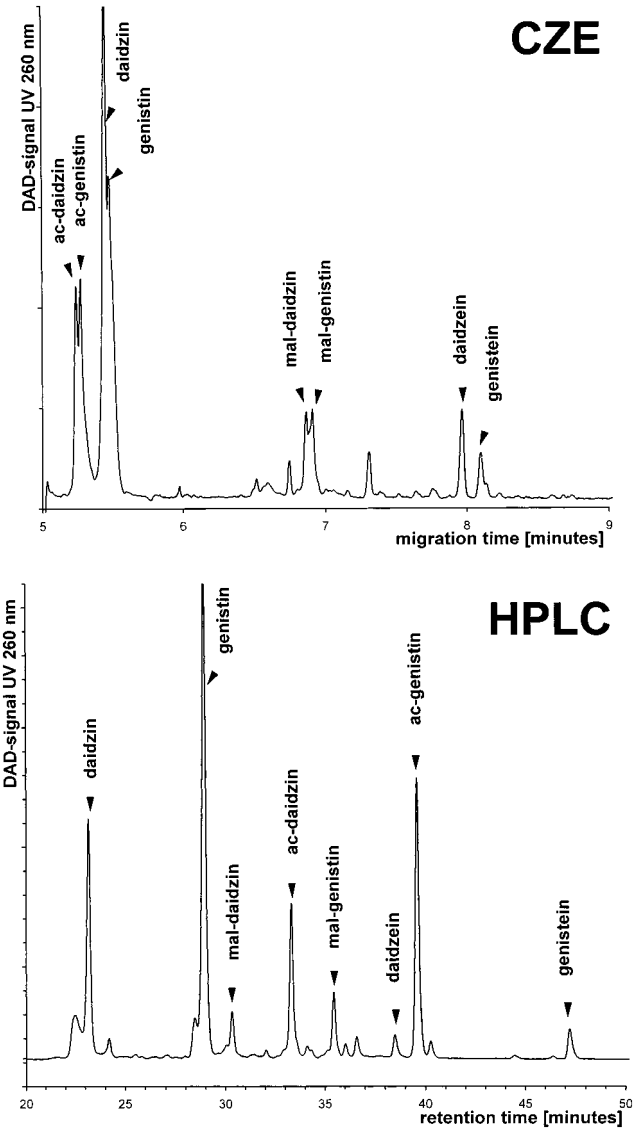

Figure 2. Comparison between CZE and HPLC separation of 8 isoflavones in a toasted soy flour (mal, 6″-*O*-malonyl derivative; ac, 6″-acetate derivative).

glucosides, aglycones. Daidzein derivatives elute earlier than genistein derivatives. Since the inner diameter of the HPLC detection cell is 11 mm, in contrast to 50 or 75 μm with the CZE, the HPLC method is due to the rule of Lambert−Beer clearly more sensitive. The detection limit for the HPLC method was about 0.01−0.03 mg/L in the final solution (depending on the type of isoflavone) compared with 0.1−0.5 mg/L in CZE. The detection limits have been determined by a method of the German DIN (Deutsches Institut für Normung e.V.).

HPLC/MS Measurements. The identity of the 6″-*O*-malonyl and 6″-*O*-acetyl derivatives in the HPLC run was established by mass spectrometry (HPLC thermospray MS coupling), because standards were not available. We measured extracts from soybeans and soy proteins both in the buffer mode (addition of ammonium acetate buffer) and in the discharge mode. In both modes the molecules tended to coordinate a proton, so the observed molecular masses were $[M + H]^+$. In the buffer mode coordination of ammonium from the buffer or potassium from the soy matrix also took place. The discharge mode was suitable for the identification of the 6″-*O*-malonyl and 6″-*O*-acetyl derivatives, whereas

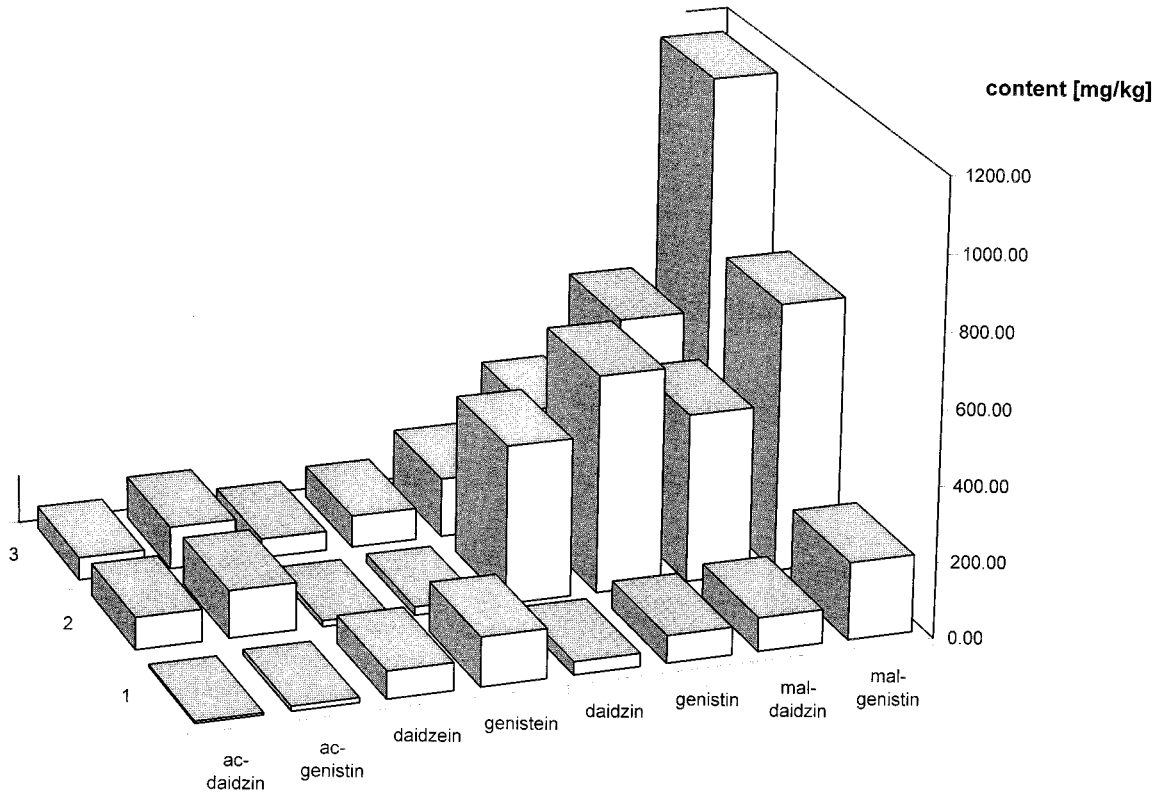

Figure 3. Different patterns of isoflavones in soy proteins (ac, 6″-*O*-acetyl glycoside; mal, 6″-*O*-malonyl glycoside).

fragmentation of the high molecular masses was predominant in the buffer mode. In case of the glycosides and their acetylated esters, respectively, cleavages into the $[M + H]^+$ ions of their corresponding aglycones (daidzein, *m*/*z* 255; genistein, *m*/*z* 271) and glucose ($[M + NH_4 - H_2O]^+ = 180$) or acetylated glucose (*m*/*z* 222) were observed. The sugar moiety of the glycosides was lost while the aglycones were stable. In most cases the base peak was the aglycon. The malonyl derivatives converted into acetyl compounds by decarboxylation or straight to the aglycones. By evaluating the mass spectra of each peak in the chromatogram and the single ion monitoring, it was possible to identify 8 of the 12 major soy isoflavones. The reliable identification of glycitein and its derivatives was not possible using mass spectrometry because of the low concentration range of these compounds. Standard material of glycitein was not available.

Further preliminary tests were performed with an electrospray interface and an ion trap mass spectrometer, which enables one to conduct MS/MS experiments. Under these conditions the isoflavones tended to lose a proton generating the masses $[M - H]^-$ (daidzein, *m*/*z* 253; genistein, *m*/*z* 269). This is in line with the results of Aramendia et al. (1995). We measured anions in the negative mode with a voltage of −5 kV. The glycosides formed adducts of $[M - H + 46]^-$ and of $[M - H + 60]^-$ with the electrospray. From these, daughter masses of $[M - H]^-$ (daidzin, *m*/*z* 415; genistin, *m*/*z* 431) could be liberated. This was also observed by Barnes et al. (1998).

UV Spectra. We recorded the UV spectra of the isoflavone standards with a photodiode array detector. The isoflavones had a main maximum between 252 and 272 nm in CZE, which had a hypsochromic shift in HPLC. The spectra were influenced by both the type of aglycone and the binding of glucose in the 7-position. The aglycones have a second smaller maximum in the range of 300−340 nm, which was especially distinct in the case of CZE spectra. The spectra clearly differ between CZE and HPLC, probably because of borate as a complexing agent in the CZE buffer (glycoside spectra) and acetonitrile as an organic solvent in the HPLC eluent (aglycone spectra). The differences in the UV spectra (position and intensity of the maxima) between CZE and HPLC result in different absorption coefficients, which influence the UV response at the wavelength of 260 nm (Figure 2). However, the general shape of an isoflavone spectrum mainly depends on the type of the aglycone. Daidzein within HPLC and all daidzein glycosides in CZE and HPLC exhibit an asymmetric, hunchbacked main maximum. In contrast, genistein and its derivatives display a sharp and slim main maximum shape.

The 6″-*O*-malonyl and 6″-*O*-acetyl derivatives were assigned to their corresponding UV spectra in HPLC by considering the results of mass spectrometric measurements, the elution order, and UV spectra of previous studies, e.g. Wang and Murphy (1994). Their appearance was very close to that of the corresponding glycosides. The proposed migration order of the 6″-*O*-malonyl and 6″-*O*-acetyl derivatives in CZE (Figure 2) was deduced from the HPLC experiments by parallel measurement of different soy proteins with CZE and HPLC, considering both the UV spectra of the compounds and the different known amount of 6″-*O*-malonyl and 6″-*O*-acetyl glycosides in the proteins. It remains to be established by CE/MS, which was not available until now. Again, the UV spectra of 6″-*O*-malonyl and 6″-*O*-acetyl glycosides were very close to those of the glycosides in CZE. Acetylation of the sugar group has only a small effect on the electrophoretic mobility (McGhie and Markham, 1994). The minor decrease in the mobilities of acetyl glycosides is due to the same charge and the increase in molecular size. In contrast, the addition of

Table 1. Amount of Isoflavones (mg/kg of Protein) in Soy Proteins Calculated by Single Determination from HPLC Experiments[a]

sample	daidzin	ac-daidzin	mal-daidzin	daidzein	genistin	ac-genistin	mal-genistin	genistein	total genistein content	total daidzein content	total aglycon content
isolate 1	173	66	593	57	351	123	1282	93	1052	499	1551
isolate 2	466	94	496	18	641	139	792	24	917	606	1522
isolate 3	169	47	399	40	517	133	997	77	996	371	1367
isolate 4	270	108	277	20	558	216	606	34	822	384	1207
isolate 5	127	24	339	62	294	80	850	139	811	326	1137
isolate 6	150	37	317	16	322	84	787	34	693	288	981
isolate 7	120	20	159	88	310	63	484	226	708	252	960
isolate 8	119	39	190	34	467	133	546	79	731	226	957
isolate 9	139	43	260	18	282	91	628	34	590	259	849
isolate 10	41	21	150	47	150	68	457	114	484	159	643
isolate 11	131	26	77	31	362	70	248	74	470	163	633
isolate 12	39	7	99	81	81	14	227	144	321	158	479
concentr	33	4	6	<1	47	7	13	1	41	26	67

[a] The total aglycone content is expressed as the sum of the aglycones genistein and daidzein after converting the contents of all glycosides into their aglycones.

Table 2. Percentage of Each Isoflavone in Relation to the Total Aglycone Content

sample	daidzin	ac-daidzin	mal-daidzin	daidzein	genistin	ac-genistin	mal-genistin	genistein	total genistein content	total daidzein content	total aglycon content
isolate 1	7	2	19	4	14	5	43	6	68	32	100
isolate 2	19	3	17	1	26	5	27	2	60	40	100
isolate 3	7	2	15	3	24	5	38	6	73	27	100
isolate 4	14	5	11	2	29	10	26	3	68	32	100
isolate 5	7	1	15	6	16	4	39	12	71	29	100
isolate 6	9	2	16	2	21	5	42	3	71	29	100
isolate 7	8	1	8	9	20	4	26	24	74	26	100
isolate 8	8	2	10	4	30	8	30	8	76	23	100
isolate 9	10	3	15	2	21	6	39	4	69	30	100
isolate 10	4	2	12	7	14	6	37	18	75	25	100
isolate 11	13	2	6	5	36	6	20	12	74	26	100
isolate 12	5	1	10	17	10	2	25	30	67	33	100
concentr	30	4	4	<1	44	6	10	2	62	38	100

a charged substituent like malonate to the sugar group has the opposite effect to acetylation (McGhie and Markham, 1994). The effect caused by introducing further negative charge seems to overcompensate the increase in molecular mass. Since the electrophoretic mobilities of the acetyl glycosides is increased, they were detected later than the unmodified glycosides. The UV spectra in the food samples were always used as an additional proof for the identity of the isoflavones. In the low concentration range, no characteristic spectra of the target compounds could be obtained. The presence of soy in meat products was only regarded as certain following two independent proofs of the result: the retention times in HPLC and CZE or the retention time in HPLC plus the DAD spectra.

Soy Isolates. Different soy products were measured for their isoflavone pattern, particularly soy flour, soy protein concentrate (70% protein), and soy protein isolate (90% protein). According to Wang and Murphy (1994), the isoflavone content even in soybeans themselves is extremely variable. The pattern mainly depends on the variety of soybean, the growth conditions, and time of harvesting. Additionally changes in the bulk ratio of isoflavones in soy protein isolates may be due to thermal treatment during manufacture. The 6″-*O*-malonyl derivatives are especially sensitive to heat and very unstable. They can be easily transformed into glycosides or into 6″-*O*-acetyl forms by decarboxylization. The degradation can progress further to the aglycones of the isoflavones, but the aglycones themselves are largely insensitive to heat. Figure 3 demonstrates the large differences in the patterns of three different soy proteins.

Large amounts of free aglycones along with low contents of 6″-*O*-malonyl derivatives indicate a thermal treatment. The protein isolate 1 has a low isoflavone content with relatively large amounts of aglycones, which points to the fact that it has been heated during manufacture. The isolates 2 and 3 were rich in glycosides with large differences in their relative amounts, especially of 6″-*O*-malonyl derivatives. The differences in the patterns can provide characteristic information about the identity of a soy protein, its previous processing, or the common characteristics of two proteins. A careful extraction procedure was used to avoid degradation of isoflavone components which would have changed the pattern. Especially in soy concentrate it is possible to find very low quantities of isoflavones because in some processes the sugar fraction is separated by leaching the defatted raw material with 50−80% aqueous ethanol, which also extracts the isoflavones (Sipos, 1982).

If the results of the isoflavonoid determination in various soy products are compared, it was obvious that the HPLC method was superior in terms of repeatability. Table 1 shows the results from HPLC. It gives the quantitative amounts of isoflavones in 12 different protein isolates and one protein concentrate. As standards of 6″-*O*-acetyl and 6″-*O*-malonyl derivatives were not available, the amounts of these substances were calculated on the basis of a calibration using the corresponding glycosides. Table 2 illustrates the different patterns of the soy proteins. To take the differences

in the molecular masses into account, the figures of each isoflavone were first expressed as their corresponding aglycones (conversion of glycosides into their aglycones) and then put in relation to the total aglycone content.

The moderate reproducibility of migration times in CZE and the narrow detection range of 4 min for 8 isoflavones led to a larger variation of the results. The results of CZE and HPLC were similar to each other, but there were also some disagreements. One reason for the differences might be caused by integration problems in CZE with peaks migrating close to each other (e.g. 6″-*O*-malonyldaidzin and 6″-*O*-malonylgenistin) as well as the more probable overlapping with matrix constituents during the short separation time.

Genetically Modified Soy. Furthermore we wanted to check whether the isoflavone pattern in soybeans was influenced by the genetic modification of the glyphosate-tolerante soybeans. Since isoflavones are derived from the secondary metabolism of plants, their production could perhaps be influenced by the addition of the new glyphosate tolerante enzyme 5-enolpyruvylshikimate-3-phosphate synthase. A number of conventional soybeans of different origin and genetically modified soybeans were examined for their isoflavone patterns. Experiments with both modified and conventional beans grown under very similar conditions were carried out to enable a comparison. In line with the results of Monsanto, no significant changes in the isoflavone patterns were noticed until now (Padgette et al., 1996).

Meat Products. It is customary to include soy protein in processed meat products. The required alteration in functional properties can be achieved by the addition of only 1−5% of soy protein to emulsified meats and coarse-ground meats. The experiments included some model sausages produced by industrial manufacturers with different defined percentages of soy proteins and corresponding sausages without soy. We chose ham sausages, liver sausages, high heated canned sausages, and strongly seasoned salami as exemplary test products. Additives of soy protein isolates in those sausages could clearly be detected with soy contents varying from 0.1% to 6%. The proposed method was also suitable for all meat products from the routine food control. In the course of our investigations we analyzed three cooked bacons from German companies and found quite small amounts of isoflavones. Actually, soy protein can be introduced into unprocessed muscle tissue by injection together with pickling brine. In CZE the daidzein peak and in particular both glycoside peaks in the front part of the electropherogram can be overlapped by other interfering peaks from the matrix. Furthermore the CZE was sensitive to adsorption of fat and protein from meat matrix on the inner surface of the fused silica capillary. In these cases a dramatic decrease of the EOF was observed. Figure 4 shows a comparison between electropherograms of sausages with and without soy.

Although the above example demonstrates matrix interferences, the peaks of the glycosides and the genistein peak clearly prove the presence of soy. Even in the sausage without a soy addition an unknown peak was present at the same migration time as daidzein. In the sausage with a soy addition a comigration was observed, which could be detected by the DAD-UV spectra, which were different. This demonstrates again the necessity of a diode array detection system in CZE. Alternatively, it was also possible to separate the peaks by polyamide SPE. The minimum soy protein contents

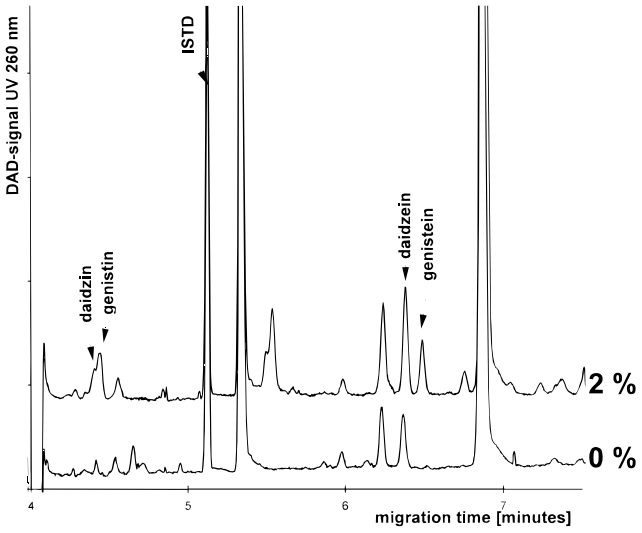

Figure 4. CZE: Electropherograms of extracts from two ham sausages (Rheinische Schinkenwurst) containing 0% and 2% soy protein.

which can be detected by CZE after a simple extraction procedure were in the range of 1%. The use of HPLC was more suitable than CZE for the detection of small amounts; it was less dependent on matrix effects and clearly more sensitive. Additives of 0.1% of isolated soy protein could be detected in industrially manufactured sausages. For meat products with a soy protein content below 0.5% the more time-consuming cleanup procedure (SPE) with polyamide was necessary.

Further Foodstuff. The method was also tested for its applicability to various other matrixes such as cheese, cakes, waffles, pastries, and soy fats. The proposed method was suitable for all kinds of foodstuff containing soy flour or soy protein. In a vegetable matrix, detection of isoflavone metabolites can more often be interfered by other polyphenols. To ensure the reliability of the results, the use of a photodiode array detector is obligatory. Although the isoflavones occur only in a few plant species, it is in fact important to know about and to consider the whole pattern of isoflavones in soy products. Through this, single isoflavones from spices, for example, cannot feign an addition of soy, because the patterns differ fundamentally between different Fabaceae plants. CZE separation was a problem with cheese as the fat and protein content affected the EOF. In this case, a SPE clean up of the target compounds was necessary prior to CZE. The HPLC separation remained unaffected. In soy lecithin and soy raw oil, we found small amounts of isoflavone aglycones. However, the detection of soy oil in margarine failed because after refinement and possibly a hydrogenation the products did not contain any detectable isoflavones.

Quantitative Measurements. A quantitative estimate of the percentage of soy in an analyzed food could only be made under certain conditions because of the great variation of isoflavone content in soy products. The genistein content in sausages with 1% soy isolate varied between 0.2 and 2 mg/kg; the total isoflavone quantities, between 0.5 and 15 mg/kg. The minimum detection level for soy protein in meat products depended on the content of isoflavones in the employed protein. If the soy protein used was available, then the content of soy in a meat product was easily calculated. Upon that

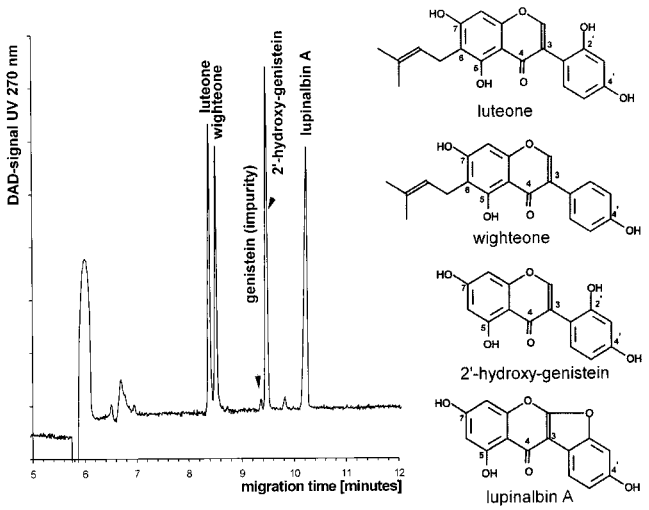

Figure 5. Chemical structures and capillary electrophoretic separation of lupin isoflavones (standard).

calculation, factors that should be considered include the possible degradation of 6″-*O*-malonyl and 6″-*O*-acetyl derivatives and also the transformations of glycosides into aglycones during the manufacture process of the meat product. If possible, the protein used should be analyzed together with the meat product in question. Therefore, food control authorities should routinely seize the protein used in the meat product alongside the collection of that product at the factory.

2. Detection of Lupin Protein. Like soy, the lupin plant belongs to the family of Fabaceae. The method development followed the same route as the soy detection. In leaves and roots of lupins, genistein and some isoflavonoides with prenyl groups or further O-heterocyclic rings were detected by Ingham et al. (1983) and Tahara et al. (1989). We analyzed standards of these isoflavones including genistein, 2′-hydroxygenistein, luteone, wighteone, and lupinalbin A using both CZE and HPLC. Gagnon et al. (1992) separated lupin isoflavones from cell cultures using HPLC. The elution order was in line with our investigations. We increased our gradient of organic eluent gradually to achieve better separation of our target compounds within the food matrix. 2′-hydroxygenistein was eluted first, followed by genistein, lupinalbin A, luteone, and wighteone. The separation of the lupin standards could also be achieved in a borate buffer by CZE within 12 min. The peaks were baseline separated. Figure 5 shows the chemical structures and the capillary electrophoretic separation of lupin isoflavones.

The UV spectra again clearly differ between CZE and HPLC. In CZE the lupin isoflavone aglycones have two maxima like daidzein and genistein. The first maximum appears between 267 nm (lupinalbin A) and 275 nm (wighteone), whereas the second maximum of a little less intensity is between 331 (2′-hydroxygenistein) and 356 nm (lupinalbin A). In HPLC, the UV spectra have one dominant maximum between 254 nm (lupinalbin A) and 265 nm (wighteone) and one to three further subsidiary maxima or shoulders. Our mass spectrometric measurements revealed that both in discharge and buffer mode $[M + H]^+$ was the main mass with all lupin isoflavones. In line with previous studies the fragment of wighteone *m/z* 283 $[M + H - C_4H_8]^+$ was observed, though it had a low intensity (Pantry et al., 1988).

We have tested lupin seeds and lupin proteins for the presence of these specific isoflavones. Our studies showed that only genistein and 2′-hydroxygenistein were present in both the seed and isolated protein. We used these as main indicating substances for the detection of lupin protein in meat products. The total amount of these isoflavonoids in lupin is much smaller than in soy. The amounts were in the range of 3−5 mg/kg of genistein and 1−5 mg/kg of 2′-hydroxygenistein. Because of such low quantities the cleanup procedure with polyamide (PA-SPE), already developed for soy, had to be employed for lupin detection in meat products in principle. The standards of 2′-hydroxygenistein, luteone, and wighteone were eluted using the same conditions as for soy. Only lupinalbin A needed a mixture of acetone and water (2 + 1, v + v) to be eluted. The PA-SPE ensures that the chromatographic separation of a cleaned up lupin protein extract exhibits mostly peaks of polyphenolic compounds. This was confirmed by the UV spectra. Actually, the majority of peaks had two typical absorption maxima as experienced in the analysis of other flavonoids, e.g. flavones (Engelhardt and Galensa, 1997). During the qualitative evaluation we took unknown peaks with a typical UV spectrum of polyphenolic compounds into account. Their fingerprint pattern was additional proof of the presence of lupin. Figure 6 shows an HPLC separation of a lupin protein after PA-SPE cleanup together with selected UV spectra.

Because of the small amounts of isoflavones in lupin protein, unlike soy, it was not possible to detect lupin as a trace ingredient in meat products. The use of HPLC was superior to CZE because of its greater sensitivity. For instance, experimental sausages (Kalbslyoner) containing 5% lupin protein were at the limit of detection using CZE. With HPLC, lupin could clearly be detected in the same sausage. Until recently, however, like soy there was not an immunological assay for lupin proteins. Figure 7 shows three chromatograms of sausages, one without lupin protein and the other ones with the addition of 5 and 8% of a lupin protein.

The chromatogram of the sausage containing no lupin protein shows no interfering peaks in the region of the genistein and 2′-hydroxygenistein peaks. In addition to the 2 indicators, the areas of other peaks (particularly between 12 and 15 min, at 23 and 27 min, from 30 to 33 min, and at 38 min) grew with increasing amount of lupin added.

3. Detection of Pea Protein. According to Franke et al. (1994) small amounts of polyphenols were occasionally found in different pea varieties, among them coumestrol and biochanin A. Sun et al. (1991) described the biosynthesis of the specific compound pisatin in pea plants, which is a pterocarpane derived from isoflavones. Coumestrol and biochanin A were not found in pea proteins, but our investigations using CZE and HPLC revealed the existence of pisatin in some of the tested pea proteins. The HPLC retention time for pisatin under the conditions used was 46 min. Furthermore, pisatin can also be concentrated by the PA-SPE procedure. Since pisatin appears as a neutral compound in the CZE buffer, it was impossible to separate it from other neutral compounds. The UV spectrum with two maxima at 284 and 307 nm in HPLC and one maximum at 309 nm in CZE was quite different from the isoflavone spectra. For HPLC/MS investigations using a thermospray coupling, the buffer mode was more suitable for pisatin. The main *m/z* 297 $[M + H - H_2O]^+$ together with *m/z* 315 $[M + H]^+$ and *m/z* 332 [M +

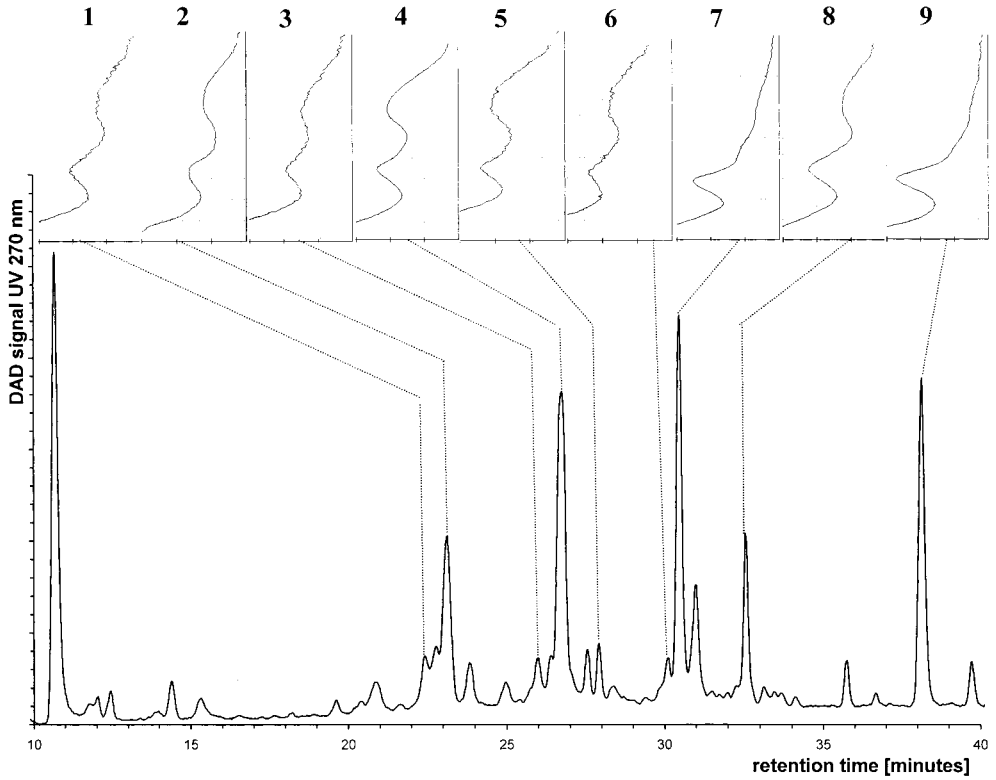

Figure 6. HPLC separation of a lupin protein after PA-SPE clean up, together with UV spectra of presumable polyphenolic compounds. Absorption maxima: (1) 270, 332; (2) 265, 330; (3) 270, 326; (4) 270, 328; (5) 270, 326; (6) 270, 328; (7) 257 (2'-hydroxygenistein); (8) 270, 331; (9) 259 (genistein) (main max is underscored).

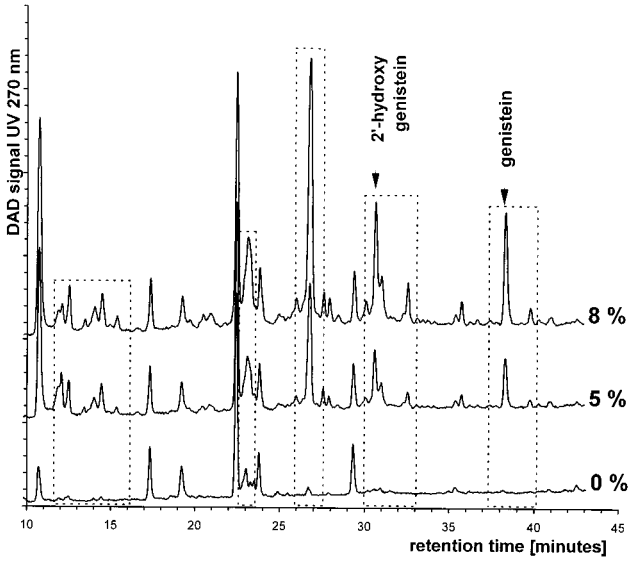

Figure 7. HPLC: Chromatograms of extracts from three sausages (Kalbslyoner) containing 0%, 5%, and 8% lupin protein.

$NH_4]^+$. If pisatin is detected in a meat product, the addition of a pea flour or protein is clearly proved. Yet, a reliable detection of pea protein is currently not possible with pisatin as the indicating substance, because it was not found in all protein samples analyzed. As a phytoalexin, pisatin is only produced in a pea plant under certain environmental conditions.

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