An Assay for Furazolidone Residues by Liquid Chromatography with Electrochemical Detection Applicable to Depletion Studies in Pigs

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A liquid chromatographic method with electrochemical detection in the reduction mode has been adapted for the estimation of furazolidone in pig tissues at residue levels. Chromatography of standard solutions shows good linearity from 0.2 to at least 10 ng injected. The limit of quantitation in spiked blank muscle and plasma is 0.5 ppb with 70% recovery and coefficients of variation ranging from 3.7 to 8.7%. In plasma, the concentration remains unchanged for up to 4 weeks at -30 °C, but the drug disappears rapidly from muscle both at room temperature and at -30 °C. Heat inactivation of spiked muscle by microwave increases the stability on storage. After the medicated diet is fed to pigs (330 ppm), the drug accumulates rapidly in plasma, reaching steady-state concentrations of approximately 500 ppb within $^1/_2$ h, but drops overnight to below the limit of detection (0.2 ppb). The concentration in the muscle is about 3-6 times lower than in plasma. Depletion from both tissues occurs with a half-life of approximately 2 h. Furazolidone is not detected in liver and kidney at any time.

Furazolidone [*N*-(5-nitro-2-furfurylidene)-3-amino-2-oxazolidone] is a veterinary drug widely used as an aid in the treatment of enteritis in swine. Administration is oral, either as a suspension or in feed at a level of 330 ppm of complete feed.

Various toxic effects from the use of furazolidone have been reviewed (Ali, 1983), and suspected toxicity in pigs, evident as a nervous syndrome, has been reported (Borland, 1979). Reference has been made to carcinogenicity in mice and rats after long-term administration (Ali, 1983) and to high mutagenic activity employing *Salmonella* (McCalla and Voutsinos, 1974). In view of these toxic effects, the possibility of residues of the drug or its metabolites in meat for human consumption is a major concern.

Studies using ^{14}C-labeled furazolidone in swine have indicated that the main excretion pathway for this drug is the urine (Vroomen et al., 1986b; Tennent and Ray, 1971). The half-life appears to be 2.6 h after a single dose, but radioactivity can still be detected in muscle tissue after 14 days. Feeding trials in swine using unlabeled drug (Winterlin et al., 1984; Vroomen et al., 1986b) have also confirmed that unchanged furazolidone is rapidly depleted from tissues. No furazolidone residue was found in muscle tissue by methods sensitive to 0.5 ppb.

A method has recently been developed in our laboratory for the assay of dimetridazole residues and its metabolites in swine tissues and plasma at 0.5 ppb (Carignan et al., 1988a,b). This method is applicable with minor variations to other nitro compounds, including furazolidone. The preliminary studies reported here were undertaken to verify the usefulness of this method in determining the elimination of furazolidone after oral administration in feed at the recommended dose level of 330 ppm.

MATERIALS AND METHODS

Chemicals and Supplies. Furazolidone was obtained from Sigma. Acetonitrile was HPLC grade (Baker). Water was purified by the Milli-Q four-bowl system (Millipore). All other solvents and chemicals were reagent grade. Dichloromethane was freshly redistilled before use. *n*-Hexane was further purified by stirring 500 mL with 100 mL of concentrated sulfuric acid overnight and washing three times with water. The chromatographic mobile phase consisted of aqueous ammonium acetate (0.067 M, pH 5.0) and acetonitrile (75:25).

A stock solution of furazolidone was prepared in acetonitrile (1 mg/mL). Working standard solutions of 1-1000 ng/mL were prepared by diluting aliquots of the stock solution in mobile phase.

Blank pig muscle was obtained from an untreated animal. The tissue was excised immediately after exsanguination, divided into 50-100-g portions, and stored at -30 °C for approximately 6 months.

Feed. The medicated feed was prepared by first making a premix of 4.95 g of furazolidone (Sigma) in 10 g of corn starch and then mixing it with 15 kg of drug-free commercial pig crumbles. The final feed contained 330 ppm furazolidone.

Apparatus. The following apparatus were used: tissue homogenizer, Polytron, Brinkman, with PT-10ST generator; centrifuges, Model K, International Equipment, and Model RC-5B, Sorval, operated at 600 G and room temperature and at 4000 G and 4 °C, respectively; rotary evaporator, Rotavapor-R, Buchi; wrist action shaker, Model 75, Burell; low actinic yellow fluorescent tubes, Phillips F40 GO-40W.

The chromatographic system consisted of two pumps (Kratos 400 equipped with start/stop remote control), pneumatic actuated switching valve (Rheodyne) controlled through a data acquisition and control system (Isaac 42A), Appligration II software (Dynamic Solutions Corp.), and an autosampler (Spectra-Physics SP8780-XR). The electrochemical detector (Coulochem 5199A, equipped with a screened wall jet cell and a gold-mercury electrode) was operated at -0.8 V vs the Coulochem reference electrode (approximately -0.5 V vs Ag/AgCl) at full gain and a response time of 10 s. Two deoxygenerators, consisting of coils made of Teflon tubing (0.3-mm i.d., 0.15-mm wall, and 15- or 30-m length, respectively) were maintained under a vacuum (<0.01 mmHg). Chromatography was performed at room temperature with two columns (Spheri-10 RP-18, 4.6 × 250 mm, Brownlee; Ultrasphere C_{18}, 5 μm, 4.6 × 150 mm, Beckman). The mobile phase, pumped at a flow rate of 1 mL/min, was continuously sparged with helium. Stainless steel tubing was used throughout.

Treatment of the Animals. Female disease-free weanling pigs, about 4 weeks old, weighing 20-25 kg and free from exposure to any medication, were used for the study. They were

0021-8561/90/1438-0716$02.50/0 Published 1990 by the American Chemical Society

housed in pens furnished with raised, plastic grid floors to facilitate cleaning and reduce coprophagia. The food bowls were filled at 7 a.m. and again at 3 p.m., and water was available at all times. Animals were held on nonmedicated feed for 8 days for acclimatization before starting the experiment.

Experiment A. One pig was cannulated (aorta), and after 4 days of convalescence its diet was changed to the medicated feed. Blood was taken prior to the change in diet (0 h) and at appropriate intervals thereafter. All blood samples were collected in heparinized containers (14.3 U/mL) and centrifuged within $^1/_2$ h. The separated plasma was stored frozen at -30 °C. Medication was continued for 14 days, and daily blood samples were taken just before and 2 h after the morning feeding to monitor furazolidone level. A series of blood samples was also taken during steady state, on day 9 of medication. On day 15, depletion of the drug was studied by giving the animal medicated feed for 2 h in the morning and then changing the diet to the control feed. Blood samples were taken just prior to change in diet and at appropriate times to follow the depletion. The pig was then returned to medicated feed for another 5 days. It was then killed 2 h after morning feeding by severing the carotid artery under total anesthesia induced by iv pentothal. Blood, muscle, kidney, and liver samples were collected.

Experiment B. Six pigs, no. 2–7, were placed on medicated diet for 8 days. On the ninth day the animals were allowed to eat medicated feed for 1.5 h in the morning, the pens and bowls were then cleaned, and control feed was given (0 h). The pigs were killed at 0.5, 1.25, 2.5, 4.5, 6.5, and 24 h with total anaesthesia followed by exsanguination as above. Blood was collected in a heparinized flask and centrifuged within 30 min. Plasma and packed red blood cells were stored separately at -30 °C until analysis. Tissue samples were chilled on ice for transport to the laboratory. A portion was processed immediately, and the remainder was stored in plastic bags at -30 °C.

Analytical Procedure. *Sample Preparation.* Depending on the concentration of the analyte, two methods of sample preparation were employed: extraction method, using dichloromethane, or deproteinization method, using acetonitrile. All analyses were performed under low actinic light.

Extraction Method. Tissue (10 g) or plasma (10 mL), anhydrous sodium sulfate (15 g), and dichloromethane (25 mL) were homogenized for 1 min in a 50-mL disposable screw-cap polyethylene centrifuge tube; the homogenate was shaken for 5 min on the shaker and then centrifuged for 5 min at 600*g*. The supernatant was decanted, through a filter paper, into a 250-mL round-bottom flask, and the sediment was reextracted two times more. All extracts were combined and evaporated to dryness on a rotary evaporator at 35–40 °C. Excessive foaming was avoided by adding 10 mL of ethanol once or twice as needed during evaporation. Mobile phase (2.0 mL) and purified *n*-hexane (10 mL) were added to the residue, and the flask was gently swirled for 1 min, making sure that the entire flask wall was wet with solvent mixture. The aqueous phase was transferred to a small test tube (12 × 75 mm) and centrifuged for 20 min at 4000*g* and 4 °C. The clear aqueous layer was transferred to an autosampler vial.

Deproteinization Method. For plasma samples containing 10 ppb or more of furazolidone, a simplified sample preparation was used. Plasma (1.0 mL) was thoroughly mixed with 2.0 mL of acetonitrile and centrifuged for 5 min at 4000*g* and 4 °C. One volume of the clear supernatant was mixed with three volumes of water and transferred to an autosampler vial.

Chromatography. Use of the switching technique for the online deoxygenation of the mobile phase, the sample, and the column effluent has been described previously (Carignan et al., 1988a). The system involves switching the mobile phase between two main circuits. One comprises the reservoir, a pump, the injector, the first deoxygenator, a low-back-pressure column, and return to the reservoir. The other includes the reservoir, the pump, the low-pressure column, the analytical column, the final deoxygenator, the detector, and return to the reservoir. The actual operation of the system involves loading a 200-μL aliquot of sample extract (equivalent to 1 g of sample) or working standard solution into the deoxygenator of the chromatographic system, stopping the flow for 2 min to permit diffusion of the oxygen, and then resuming the flow for 2 min to transfer the aliquot on the first column. After the valve is switched, chromatography is accomplished on both columns in tandem. Excluding the first deoxygenator from the analytical circuit spares it from being subjected to excessive pressure.

Concentrations of unknown samples were calculated by comparing peak heights to those of the working standard solutions, without correcting for recovery.

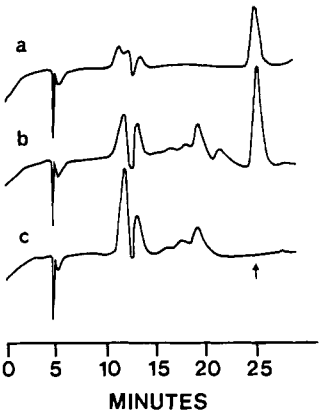

Figure 1. Typical chromatograms of furazolidone: a, standard solution (1 ng injected); b, dichloromethane extract of pig muscle tissue from animal fed medicated diet (calculated concentration, 1.6 ppb not corrected for recovery); c, dichloromethane extract of blank muscle tissue. Retention time of furazolidone: 25.1 min (arrow). Conditions: two reversed-phase columns in tandem; mobile phase, ammonium acetate buffer–acetonitrile (75:25); flow rate, 1 mL/min; detector, gold–mercury at -0.8 V vs Coulochem reference; injection volume, 0.2 mL. For other details, see text.

Table I. Recovery of Furazolidone from Spiked Blank Tissue[a]

tissue	amt added, ppb	N^b	mean rec, %	CV, %
plasma	2	2	79.9	
	10	2	78.8	
	20	1	79.7	
	30	2	75.4	
	40	2	68.7	
muscle	0.5	5	70.0	4.4
	1.0	5	71.2	8.7
	5.0	5	69.5	3.9
	10.0	5	67.1	3.7

[a] Control plasma or muscle was spiked and extracted immediately with dichloromethane. [b] Number of samples extracted.

RESULTS

Method. The method originally developed for dimetridazole by Carignan et al. (1988a) was found to be applicable to the analysis of furazolidone in the tissue tried. A hydrodynamic voltammogram for furazolidone in the mobile phase at pH 5.0 has shown a maximum response at -0.8 V, a considerably lower potential than that required for dimetridazole. This lower potential led to higher selectivity and less interference (Figure 1).

Linearity and reproducibility of the HPLC/detector system was determined by five replicate injections of standard solutions of furazolidone at 0.2, 0.5, 1, 5, and 10 ng injected. The coefficient of variation (CV) of the means varied between 2.31% at 0.2 ng and 0.49% at 10 ng. The correlation coefficient was better than 0.999; slope and intercept (±SE) of the regression were 0.331 (0.006) and -0.016 (0.010), respectively.

Recovery of the drug after dichloromethane extraction was studied by spiking blank muscle with 0.5–10 ppb drug and extracting immediately. Recoveries of 67–71% were obtained with acceptable CV (Table I). Some-

Table II. Stability of Furazolidone in Muscle and Plasma

time	storage temp, °C	furazolidone found,[a] ppb: muscle spiked[b]	muscle incurred[c]	plasma[d]
0 h		7.59	60.7	137.9
1 h	room temp	7.05		
2 h	room temp	5.14		
3 h	room temp	2.59		
4 h	room temp	2.10		
1 week	-30		19.7	143.9
2 weeks	-30		1.18	
4 weeks	-30		2.18	129.3

[a] Means of duplicate determinations. [b] Blank pig muscle kept at -30 °C for 6 months before spiking with 10 ppb furazolidone. Extracted with dichloromethane after different times at room temperature. [c] Incurred muscle from treated animal extracted with dichloromethane, fresh, or after 1–4 weeks at -30 °C. [d] Incurred plasma from treated animal with the acetonitrile precipitation method on the fresh specimen (0 h) or after frozen storage at -30 °C for 1 and 4 weeks.

what higher recoveries were obtained with blank plasma spiked with 10–200 ppb drug.

The stability of furazolidone was determined by thawing the blank muscle at room temperature, spiking it with 10 ppb of the drug and extracting it immediately, or after 1–4 h at room temperature. Similar studies were conducted with incurred plasma and muscle stored from 1 to 4 weeks at -30 °C (Table II). In incurred plasma stored at -30 °C furazolidone was found to be stable for at least 1 month. In muscle, however, it decreased rapidly both at room temperature and at -30 °C.

The stability of furazolidone in spiked muscle was increased considerably by heat denaturation of the proteins, using microwave within a few minutes after spiking. Although there was a decrease of about 50% in the amount of drug recovery from muscle spiked before, as compared to that spiked after heating, this lower recovery remained essentially unchanged during storage for several weeks at -30 °C. The loss on heating could not be accounted for by destruction of the drug by the heat per se, since aqueous solutions treated similarly proved to be stable.

Feeding Experiments. *Experiment A.* After the animal was placed on medicated feed, the drug was absorbed rapidly, reaching a maximum level about 1 h after the first feeding (Figure 2). The level was maintained with minor variations during the sampling period of 3.5 h. A minor dip in the concentration at about 2–3 h, followed by an increase within 30 min, could be observed in all profiles tested (Figures 2–4).

Day to day levels in the blood, determined by sampling the blood during 14 consecutive days 2 h after the first morning meal, were highly variable, but the largest number of samples (6 out of 14) were in the range of 510–609 ppb. Variation in plasma level during this steady-state, followed during the course of a day, showed a pattern similar to the absorption curve, including the drop in concentration at approximately 2 h, followed by a rise (Figure 3). On three occasions blood was also taken just before the morning meal. These contained no measurable quantities of furazolidone.

In order to follow the depletion of furazolidone in the plasma, the animal was given one morning meal of medicated feed and the diet was changed to control feed (0 time), available ad libitum. The plasma profile of the drug is illustrated in Figure 4. Except for the characteristic increase at 2–2$^1/_2$ h, the concentration declined rapidly with a half-life of approximately 2 h. Only trace amounts were present after 7-h withdrawal time.

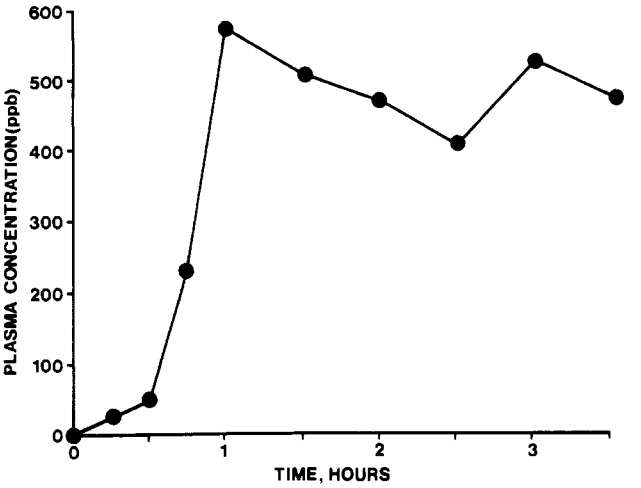

Figure 2. Accumulation of furazolidone in the plasma of pig no. 1 upon first presentation of medicated feed (0 h: 8 a.m.). Deproteinization with acetonitrile. Chromatographic conditions as in Figure 1 (values not corrected for recovery).

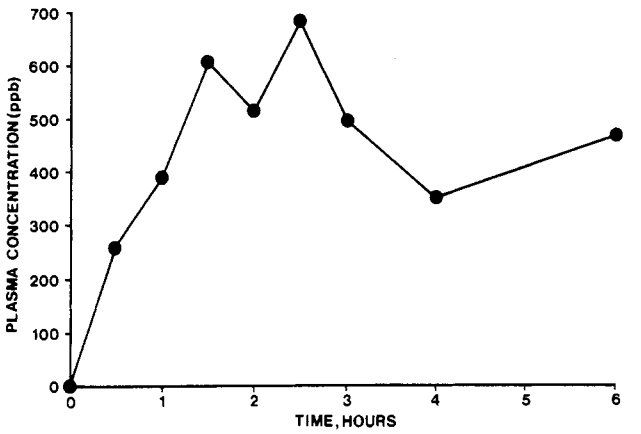

Figure 3. Furazolidone concentrations in the plasma of pig no. 1 during steady state (day 9 of medication). 0 h: Presentation of morning meal (8 a.m.). Conditions as in Figure 2.

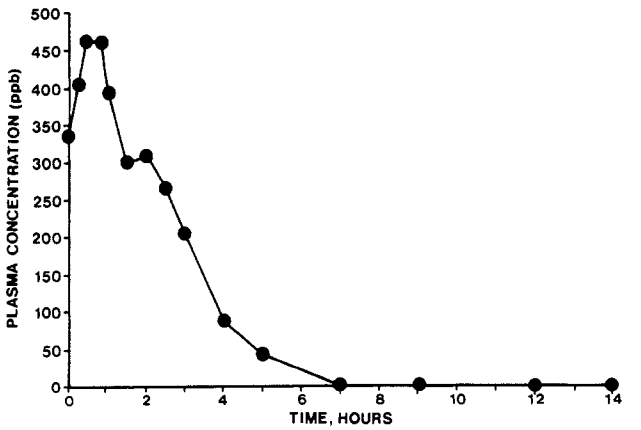

Figure 4. Furazolidone concentrations in the plasma of pig no. 1 upon discontinuation of medication. The animal was presented a morning meal of medicated feed and 1.5 h later (0 h) changed to control diet. Conditions as in Figure 2.

Experiment B. Depletion of furazolidone from the tissues was studied in six piglets. After 8 days of medication, the animals were exposed to one medicated morning meal, then changed to control diet (0 h), and killed at appropriate times (Table III). In all but the first animal (0.5 h), lower plasma concentrations were observed than expected on the basis of the results of the prelimi-

Table III. Depletion of Furazolidone from Pig Plasma and Muscle

pig no.	time,[a] h	plasma,[b] ppb	muscle,[b] ppb	ratio muscle/plasma
2	0.5	384.6	60.7	0.16
3	1.25	45.3	13.8	0.30
4	2.5	141.6	28.3	0.20
5	4.5	3.5		
6	6.5	c	<0.5	
7	24.0			

[a] Time after change to unmedicated feed. [b] Extracted with dichloromethane. [c] Not detectable (<0.2 ppb).

nary experiment (Figure 4). The anticipated dip, at about 1.25 h, was also much larger than expected. At least part of the discrepancy could be explained by a large intersubject variation: Blood taken from all six animals on day 3 of medication, 2 h after the first morning feeding, showed widely differing concentrations ranging from 138 to 325 ppb.

Drug concentrations in the muscle were about 3–6 times lower than that in plasma, but followed a similar pattern. Furazolidone was not detected in either the liver or the kidney of any of the animals.

DISCUSSION

Due to its high extinction coefficient at 362 nm, furazolidone can be detected with high sensitivity by HPLC with a UV detector. Although this method has been applied to tissues by a number of authors with varying success, the higher sensitivities obtainable by electrochemical detection warranted investigation.

The method of Winterlin et al. (1981) involves a lengthy extraction procedure. The claimed sensitivity is 0.5 ppb with recoveries in the order of 80%. This method, however, has only been evaluated on spiked blank tissues. Attempts to measure residues in incurred chicken and swine tissues were unsuccessful (Winterlin et al., 1984).

Veale and Harrington (1982) have also reported a method using HPLC with UV and electrochemical detection. Although they claim a sensitivity of 1 ppb for spiked plasma, no figures below 10 ppb have been provided for either the UV or the electrochemical detection.

The method of Vroomen et al. (1986a) is much simpler but seems to be less sensitive, at 2 ppb in muscle and 25 ppb in urine. With this method they have shown the presence of furazolidone in the blood, but not in other tissues, of pigs 2 h after feeding on medicated feed (Vroomen et al., 1986b). Although the drug disappeared rapidly, there was an accumulation of breakdown products, as shown by the use of [^{14}C]furazolidone.

While this paper was in preparation, Botsoglou (1988) described a method for furazolidone residues in eggs, sensitive to 1 ppb. The sample preparation appears complicated, and the method, although promising, has not been applied to other tissues.

The method described here seems to be applicable to depletion studies. In these preliminary studies under simulated farm conditions, pigs were fed a diet containing the recommended level of furazolidone. The animals, weighing 20–25 kg, were given approximately 2 kg of feed/day each. In spite of efforts to prevent spillage, much of this feed was wasted; thus, the actual daily dose was under 25 mg/kg. Under these conditions plasma concentrations of the drug could be followed during medication and for several hours after withdrawal. Much lower, but still measurable, concentrations were observed in the muscle.

A major concern, both from a regulatory point of view and for depletion studies, is the stability of the analyte during transport of the sample from its origin to the testing facility. With muscle tissue that has been stored frozen and then thawed and kept at room temperature, furazolidone was shown to disappear within a few hours after spiking. The drop in concentration was a great deal more rapid in incurred muscle. It was felt that this concern could be answered by stabilizing furazolidone through inactivation of the enzymes involved. As a first attempt heat denaturation by microwave was tried. This treatment did prevent loss on storage albeit after some initial loss, which could have been due to either accelerated breakdown during heating or occlusion of the drug in the denatured protein matrix. Optimizing the conditions (sample size, heating time, etc.) might reduce losses, while stabilizing the residue.

An interesting feature observed in all plasma profiles was a dip in the concentration, followed by a temporary increase about $1^1/_2$–3 h after the first morning meal. This pattern was observed on three separate occasions in the plasma of one animal (experiment A) and in the plasma and muscle of the animals in experiment B. This was not likely to be due to the eating habits of the animals, since it was also observed in the depletion phase, during a time of the day when only unmedicated feed was available to the animal. Stomach emptying or enterohepatic recirculation are possible explanations.

The method described in this paper is sensitive to better than 0.5 ppb, requires little sample preparation, and appears to be valid for incurred plasma and muscle. Although the method seems adequate, our preliminary results on depletion clearly indicate that the problem of stability should be resolved before a major study on elimination can be undertaken.

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Received for review March 28, 1989. Accepted September 26, 1989.

Registry No. Furazolidone, 67-45-8.

Determination of Tryptophan in Unhydrolyzed Food and Feedstuffs by the Acid Ninhydrin Method

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The optimum conditions for direct measurement of tryptophan in soybean meals, wheat, maize, barley, rye, sunflower, fish, and meat meals have been reported. The acid ninhydrin solution has been reacted directly with the protein matrix without any previous isolation or dissolution of the protein. Reproducibility and recovery studies showed that 10–100 μg of tryptophan-containing protein can be determined with a standard error of 2.7% or less. The decreases of tryptophan content of treated soybeans are essentially a function of the parameters applied. The remaining tryptophan contents, expressed in the percentages of the untreated one, were as follows: 81% HCl + H_3PO_4 (pH ~1.8), neutralized; 79% HCl + H_3PO_4 (pH ~2.0), neutralized; 67% HCl + H_3PO_4 (pH ~2.0), without neutralization; 63% microwave 5 min, 100 °C; 57% HCl (pH ~2.0), without neutralization.

Finding a really reproducible tryptophan assay is still a relevant task in protein research (Cuq et al., 1983; Cuq and Cheftel, 1983; Finot et al., 1982; Friedman and Finley, 1971; Friedman et al., 1984; Friedman, 1984; Friedman and Cuq, 1988; Nielsen et al., 1985a,b). It is well-known that, under the common parameters of protein hydrolysis (6 mol of HCl, 100 °C, 24 h), a considerable amount of tryptophan became destroyed because of the particular reactivity of the indole ring (Fontana, 1984).

To avoid tryptophan losses, time-consuming and tedious special hydrolysis conditions have been suggested in the literature: Several procedures are based on hydrolyses by hydrochloric acid with additives (Andersen et al., 1984; Ashworth, 1987; Felker, 1976; Gardner, 1984; Gruen and Nicholls, 1972; Matsubara and Sasaki, 1969; Ohta and Nakai, 1979; Wong et al., 1984), by organic acids (Csapó and Csapó-Kiss, 1985; Csapó and Csapó-Kiss 1986; Csapó et al., 1986; Gundel and Votisky, 1974; Liu and Chang, 1971; Penke et al., 1974; Simpson et al., 1976), or by bases (Allred and MacDonald, 1988; Buttery and Soar, 1975; Delhaye and Landry, 1986; Gorinstein et al., 1988; Huet and Pernollet, 1986; Hugli and Moore, 1972; Kirchgessner et al., 1987; Knox et al., 1970; Landry et al., 1988; Levine, 1982; Lucas and Sotelo, 1980; Nielsen et al., 1985a,b; Nielsen and Hurrel, 1984, 1985; Piombo and Lozano, 1980; Sato et al., 1984, 1987; Werner, 1986; Williams et al., 1982).

Less emphasis have been focused on the so-called direct methods that do not need the previous hydrolysis of proteins (Barman and Koshland, 1967; Chrastil, 1986; Concon, 1975; Dickman and Crockett, 1956; Friedman and Sigel, 1966; Friedman, 1967; Gaitonde and Dovey, 1970; Iizuka and Yajima, 1985; Nakae and Shono, 1984; Opienska-Blauth et al., 1963; Servillo et al., 1982; Sodek et al., 1975; Votisky, 1984; Zahnley and Davis, 1973).

As a result of earlier comparative studies, it has been stated (Friedman et al., 1984) that "the acid ninhydrin method, merits further study to assess its general applicability".

Thus, recently, exhaustive investigations have been carried out to find optimum conditions for the direct tryptophan assay to improve the acid ninhydrin method (Molnár-Perl and Pintér-Szakács, 1989).

As a result of kinetic studies (Molnár-Perl and Pintér-Szakács, 1989) it has been proven that excellent results can be achieved at 35 °C after 2-h reaction time, with 1% ninhydrin containing acidic reagent (96% formic acid/cc. HCl = 3/2; detailed composition in Materials and Methods (i) without the disturbing effect of tyrosine reported by Zahnley and Davies (1973) and (ii) without the necessity of previous isolation (Sodek et al., 1975) or dissolution (Gaitonde and Dovey, 1970) of the protein content of the sample to be analyzed.

In this paper we show the applicability of the acid ninhydrin method in determination of the tryptophan content both in intact food and feedstuffs and in differently treated soybean meals.

MATERIALS AND METHODS

Materials. Bovine serum albumin 1, human serum albumin 1, and α-chymotrypsin were obtained from Reanal (Buda-

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