suggests that S_0 is a major determinant of FBC of proteins.

LITERATURE CITED

Aoki, H; Taneyama, D.; Orimo, N.; Kitagawa, I. *J. Food Sci.* **1981**, *46*, 1192.
Brewer, J. M.; Pesce, A. J.; Ashworth, R. B. "Experimental Techniques in Biochemistry"; Prentice-Hall: Englewood Cliffs, NJ, 1974.
Briggs, D. R. *J. Biol. Chem.* **1940**, *134*, 261.
Dippold, M. W. "What's New in Products from the Soybean", Midwest Section American Association of Cereal Chemists Symposium, 1961 (Cited by Johnson, D. W. *J. Am. Oil Chem. Soc.* **1970**, *47*, 402).
Franzen, K. Ph.D. Thesis, Cornell University, 1975 (Cited by Kinsella, J. E. *CRC Crit. Rev. Food Sci. Nutr.* **1976** *7*, 219).
Hutton, C. W.; Campbell, A. M. In "Protein Functionality in Foods"; Cherry, J. P., Ed.; American Chemical Society: Washington, DC, 1981; ACS Symp. Ser. No. 147, p 177.
Johnson, D. W. *J. Am. Oil Chem. Soc.* **1970**, *47*, 402.
Karel, M *J. Food Sci.* **1973**, *38*, 756.
Kato, A.; Nakai, S. *Biochim. Biophys. Acta* **1980**, *624*, 13.
Kinsella, J. E. *Food Technol. (Chicago)* **1975**, *29* (5), 82.
Kinsella, J. E. *CRC Crit. Rev. Food Sci. Nutr.* **1976**, *7*, 219.
Lin, M. J. Y.; Humbert, E. S.; Sosulski, F. W. *J. Food Sci.* **1974**, *39*, 368.
Morr, C. V. In "Functionality and Protein Structure"; Pour-El, A., Ed.; American Chemical Society: Washington, DC, 1979; ACS Symp. Ser. No. 92, p 65.
Morris, C. E. *Food Eng.* **1980**, *52* (9), 28.
Nakai, S.; Ho, L.; Tung, M. A.; Quinn, J. R. *Can. Inst. Food Sci. Technol. J.* **1980**, *13*, 14.
Nakai, S.; Le, A. C. *J. Dairy Sci.* **1970**, *53*, 276.
Pearse, K. N.; Kinsella, J. E. *J. Agric. Food Chem.* **1978**, *26*, 716.
Perlmann, G. *Biochem. J.* **1938**, *32*, 931.
Perlmann, G.; Herrmann, H. *Biochem. J.* **1938**, *32*, 926.
Pham, A.-M. M.Sc. Thesis, University of British Columbia, Vancouver, B.C., Canada, 1981.
Pomeranz, Y. *Adv. Food Res.* **1973**, *20*, 153.
Ryan, D. S. In "Food Proteins: Improvement through Chemical and Enzymatic Modifications"; Feeney, R. E.; Whitaker, J. R., Eds.; American Chemical Society: Washington, DC, 1977; ACS Adv. Chem. Ser. No. 160, p 67.
Torgersen, H.; Toledo, R. T. *J. Food Sci.* **1977**, *42*, 1615.
Voutsinas, L. P.; Cheung, E.; Nakai, S. *J. Food Sci.* **1982**, in press.
Wall, J. S. *Cereal Foods World* **1979**, *24* (7), 288.
Wang, J. C.; Kinsella, J. E. *J. Food Sci.* **1976**, *41*, 286.
Wolf, W. J. In "Soybeans: Chemistry and Technology"; Smith, A. K.; Circle, S. J., Eds.; Avi Publishing Co.: Westport, CT, 1972.
Wolf, W. J.; Cowan, J. C. "Soybeans as a Food Source"; CRC Press: Cleveland, OH, 1975.

Received for review May 11, 1982. Accepted September 24, 1982. A part of this work was presented at the 24th Annual Conference of the Canadian Institute of Food Science and Technology, Winnipeg, Manitoba, Canada, June 21–24, 1981.

A Gas–Liquid Chromatographic Method for the Analysis of Methionine and of Methionine Sulfoxide in Proteins

Klaas D. Bos, Cornelis Verbeek, and Pier Slump*

The determination of methionine and methionine sulfoxide in feeds by gas–liquid chromatography is described. Methionine is determined as methyl thiocyanate, after reaction of the intact protein with cyanogen bromide. The indirect determination of the methionine sulfoxide involves the reduction of the sulfoxide with titanium trichloride and subsequent determination of the methionine via the cyanogen bromide method. The difference between this value for methionine and that obtained via the direct determination of the methionine without a reduction step gives the amount of methionine sulfoxide. The method was applied to a series of feed and food proteins. Some of these samples had a sulfoxide content of 10–20% of total methionine. This technique has potential for measuring nutritionally available methionine in feeds by a rapid chemical method, which does not require protein hydrolysis.

Methionine is an essential amino acid that is the limiting factor in several feed proteins (Woodham, 1978). Part of the methionine in certain proteins may be present in an oxidized form, often the sulfoxide, but, in strongly oxidized proteins, methionine sulfone may be present also. In recent years, several papers have been published on the biological availability of methionine in relation to the presence of methionine sulfoxide and sulfone (Slump and Schreuder, 1973; Gjøen and Njaa, 1977; Kuzmicky et al., 1977; Sjöberg and Boström, 1977; Cuq et al., 1978; Ellinger, 1978). Methionine sulfone has been found to be biologically nonavailable (Kuzmicky et al., 1977; Sjöberg and Boström, 1977), and the availability of methionine sulfoxide is generally expected to be less than that of methionine (Gjøen and Njaa, 1977; Kuzmicky et al., 1977; Cuq et al., 1978). A good analytical method for the determination of methionine sulfoxide is of great value for the interpretation of the results of feeding experiments. Methionine sulfoxide in proteins has been determined by two *direct* methods, one involving alkaline hydrolysis and determination of the methionine sulfoxide by automatic amino acid analysis (Neumann, 1967) and the other involving a reaction with acetic anhydride and determination of the formaldehyde formed by a color reaction with chromotropic acid (Lunder, 1972).

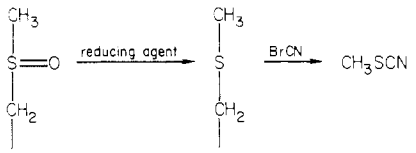
Alternatively, some *indirect* methods may be used to determine methionine sulfoxide in proteins. For example, reaction of a protein with iodoacetic acid will convert the methionine residues to sulfonium salts. Subsequent oxidation with performic acid results in the formation of methionine sulfone from the methionine sulfoxide, if present. Upon hydrolysis the methionine sulfone is determined by ion-exchange chromatography (Neumann, 1967; Slump and Schreuder, 1973; Sjöberg and Boström, 1977). The direct method, involving alkaline hydrolysis, may result in low recoveries of methionine sulfoxide (Neumann, 1967; Lunder, 1972). The indirect method with iodoacetic acid is complicated and time consuming and is

Institute CIVO-Analysis TNO, P.O. Box 360, 3700 AJ Zeist, The Netherlands.

0021-8561/83/1431-0063$01.50/0

therefore not very suitable for routine analysis.

The indirect determination of methionine sulfoxide suggested by Ellinger and Smith (1971) and by Ellinger (1978) seemed to be a more attractive procedure, but these authors did not publish the experimental details. We have developed this suggested method and the results obtained are described herein. The technique involves the reduction of the methionine sulfoxide to methionine and the determination of the total methionine via the cyanogen bromide reaction.



This cyanogen bromide reaction is an improved version of the procedure described by Ellinger and Duncan (1976). Many reagents may be used for the reduction of a sulfoxide to the parent sulfide (Block, 1978), and in this study, titanium trichloride turned out to be the most suitable. The methyl thiocyanate formed in the cyanogen bromide reaction is determined by gas–liquid chromatography, and the value obtained after reduction of the protein is the total of the contents of the methionine and the methionine sulfoxide initially present. A direct analysis of the sample by the cyanogen bromide method without a reduction step gives the value for the nonoxidized methionine. The difference of the two values obtained equals the amount of methionine sulfoxide present. Recently, another indirect method for the determination of methionine sulfoxide has been published (Njaa, 1980). That method is based upon hydrolysis of the protein with barium hydroxide and determination of the methionine by a colorimetric technique, using an iodoplatinate reagent. Methionine sulfoxide is obtained by difference, by carrying out one determination without and one determination with previous reduction of a portion of the sample with titanium trichloride.

MATERIALS AND METHODS

Reagents. Cyanogen bromide and formic acid (98%) were purchased from J. T. Baker Chemicals, Deventer, Holland, and titanium trichloride (15% solution in water) was obtained from Merck, Darmstadt, West Germany (product no. 10789). Tin dichloride was supplied by BDH Chemicals, Poole, England, and ethyl thiocyanate was purchased from EGA-Chemie, Steinheim, West Germany. A stock solution of cyanogen bromide was prepared by dissolving 13 g of cyanogen bromide in 100 mL of 70% formic acid. This solution was stored at 4 °C in the refrigerator and renewed twice monthly. A solution of tin dichloride in water was prepared by dissolving 12.5 g of $SnCl_2 \cdot 2H_2O$ in 100 mL of water and acidified with 1.0 mL of 6 N hydrochloric acid. The standard solution of ethyl thiocyanate was prepared by mixing 40 mL of formic acid, 60 mL of water, and 250 μL of ethyl thiocyanate. Cyanogen bromide and ethyl thiocyanate are highly toxic substances, and all handlings with these compounds should be performed with caution in the fume cupboard.

Protein Samples. The samples were obtained mainly from feedstuff factories and from food industries and were milled with a water-cooled analytical mill, until the resulting powder passed through a sieve of 20 mesh. Care must be taken to ensure that the sample does not become inhomogeneous due to electrostatic forces. The oxidized casein was prepared by the careful oxidation of commercially available casein with hydrogen peroxide at pH 8 and 50 °C (Cuq et al., 1978). ANRC reference casein was obtained from Humko Sheffield, New Jersey.

Apparatus. A Tracor Model MT 160 gas chromatograph equipped with a flame photometric detector and a flame ionization detector was used. The column was a 4 mm × 2.5 m glass tube packed with uncoated 60–80-mesh Chromosorb 101. Rates of gas flow to the FDP detector were adjusted at the following values: H_2, 100 mL/min; air, 80 mL/min. By use of nitrogen carrier gas at a flow rate of 40 mL/min and a column temperature of 150 °C, the retention time for MeSCN was found to be about 12 min and that for EtSCN about 19 min. The liner (1.3-mm i.d.) was cleaned after a series of 50 injections. Amino acid analyses by ion-exchange chromatography were performed on a Beckman "Multichrom B" analyzer.

Determination of Methionine by the Cyanogen Bromide Reaction. A protein sample (300 mg or less) containing a maximum of 3 mg of methionine was accurately weighed and transferred to a glass tube with a ground glass joint. Formic acid (98%, 3.5 mL) was added on a vortex mixer. The resulting solution or suspension was diluted with 1.5 mL of water, and 0.5 mL of the cyanogen bromide solution was added on a vortex mixer. The glass tube was stoppered and placed in a water bath at 30 °C for 4 h. Tin dichloride solution (1.0 mL) was added on a vortex mixer, followed by 1.00 mL of the ethyl thiocyanate standard solution. If a precipitate is present, the mixture is centrifuged at 2000 rpm for 5 min. The supernatant is transferred to another tube and homogenized on a vortex mixer. A 1.0-μL sample of the solution is injected into the gas chromatograph for analysis.

Reduction of Protein-Bound Methionine Sulfoxide with Titanium Trichloride. A protein sample (300 mg or less) containing a maximum of 3 mg of methionine was accurately weighed and transferred to a glass tube with a ground glass joint. Formic acid (98%, 3.5 mL) was added on a vortex mixer. The titanium trichloride (100 μL of a 15% solution) was added and vortexed also. The reaction tube was provided with a reflux condenser and placed in an oil bath at 120 °C for 2 h. Some pieces of carborundum were added to prevent bumping. The mixture was cooled to room temperature and 1.5 mL of water was added on a vortex mixer, followed by 0.5 mL of the cyanogen bromide solution. The methionine content was determined by using the cyanogen bromide procedure described above.

Gas Chromatographic Analysis. All samples were analyzed by using the operating conditions mentioned previously. The two signals, one from the flame photometric detector and the other from the flame ionization detector, were recorded by a dual-channel recorder. The chromatogram from the FID was used to calculate the amount of MeSCN via the peak areas of the MeSCN and the standard EtSCN. Only a very small error is made if the peak heights instead of the peak areas are used for the calculation of the MeSCN. The FPD chromatogram was used to check if other sulfur-containing volatiles might interfere with the MeSCN and EtSCN.

Ion-Exchange Chromatography. The proteins were oxidized with performic acid and hydrolyzed with hydrochloric acid. The methionine was determined as methionine sulfone (Slump and Schreuder, 1973).

RESULTS AND DISCUSSION

Reduction of Methionine Sulfoxide. The reduction of the methionine sulfoxide in oxidized casein was carried out by using tin dichloride in hydrochloric acid (Ho and Wong, 1973b), cobalt dichloride with sodium borohydride (Chasar, 1971), or titanium trichloride (Gawargious, 1971; Ho and Wong, 1973a) as reducing agents. The latter turned out to be the best reducing agent in terms of reaction rate and yield at room temperature. The most

Table I. Methionine Content[a] As Found by the Cyanogen Bromide Method after Reduction with Varying Amounts of Titanium Trichloride

	added $TiCl_3$ solution, μL				
product	20 + 10	50	100	100 + 50	200 + 100
soybean meal (Brazilian)	0.56	0.62	0.62	0.60	0.58
casein, oxidized	1.43	2.57	2.58	2.57	2.56
gluten, wheat	1.06	1.12	1.11	1.13	1.03
whey powder	0.13	0.20	0.21	0.21	0.20
soy isolate, Promine D	0.43	1.07	1.05	1.07	1.02

[a] In gram(s) per 100 g of product.

Table II. Unoxidized Methionine[a] and Methionine plus Methionine Sulfoxide[a] in a Gluten and in a Soy Isolate, Analyzed on Two Days

		BrCN method without reduction		BrCN method after reduction with $TiCl_3$	
		mean[b]	SD	mean[b]	SD
gluten (wheat)	day 1	0.768	0.011	1.08	0.012
	day 2	0.784	0.012	1.09	0.016
soy isolate (Promine D)	day 1	0.172	0.005	0.980	0.040
	day 2	0.176	0.006	0.978	0.020

[a] In gram(s) per 100 g of product. [b] Average of five replicates.

Table III. Recovery of Methionine Sulfoxide (Added as Oxidized Casein) from a Gluten and from a Soy Isolate

(A) Gluten, Wheat			
wheat gluten, g	added oxidized casein, g	av % recovery[a]	SD
0.200	0.005	99.8	1.30
0.200	0.015	99.4	1.34
0.175	0.025	100.2	1.10
	total av recovery:	99.8	

(B) Soy Isolate, Promine D			
soy isolate, g	added oxidized casein, g	av % recovery[a]	SD
0.200	0.010	97.6	3.13
0.170	0.035	99.8	1.30
0.130	0.055	98.2	0.44
	total av recovery:	98.5	

[a] Average of five replicates.

suitable solvent is 100% formic acid, and the great advantage of this solvent is that it is also used in the subsequent reaction with cyanogen bromide. The minimum quantity of titanium trichloride solution needed for total reduction of the sulfoxide was determined in a series of experiments (Table I).

Cyanogen Bromide Reaction. A modified version of the procedure described by Ellinger and Duncan (1976) was used. The published method was not useful for the analysis of leguminous seeds because sulfur-containing volatiles were formed. These volatiles interfered with the methyl and ethyl thiocyanate peaks in the gas chromatogram. The use of tin dichloride instead of sodium thiosulfate for the destruction of excess cyanogen bromide resulted in gas chromatograms without interfering peaks (Figure 1). A temperature of 30 °C for the cyanogen bromide reaction has the advantage that the reaction time may be decreased from 16 to 4 h.

Accuracy and Reproducibility. The reproducibility of the method was applied for two protein samples, a wheat gluten and a soy isolate, that were analyzed on two different days (Table II). Percentage recoveries of methionine sulfoxide added as oxidized casein to a wheat gluten and a soy isolate, are presented in Table III. The methionine sulfoxide had to be added in a protein-bound form because the cyanogen bromide method is only suitable for the analyses of protein-bound methionine (Ellinger and Duncan, 1976). The accuracy of the cyanogen bromide/titanium trichloride method will largely depend upon the limitations that are inherent with the cyanogen bromide reaction. Free methionine gives only a very low yield of methyl thiocyanate. Two neighboring methionine residues in a protein chain or methionine in an N-terminal position give a low yield of methyl thiocyanate also (Ellinger and Duncan, 1976). In our opinion, these limitations are not a serious drawback of the described method for the analysis of methionine in many feed and food proteins. The method is, however, useless for the analysis of feeds enriched with free methionine. Great care should be taken to obtain a fine milling of the protein samples since complete dissolution of the protein particles in the formic acid is necessary for the cyanogen bromide reaction, as well as for the titanium trichloride reduction, to run to completion.

Methionine Sulfoxide Level in Food and Feed Proteins. The reduction method developed with titanium trichloride in combination with the cyanogen bromide reaction was used to analyze several protein-containing materials. The results are given in Table IV. For comparison, the results of the determination of methionine by ion-exchange chromatography are given in the last column

Table IV. Methionine[a] and Methionine Sulfoxide[a] Levels in Feed and Food Proteins

product	unoxidized Met, I (BrCN method without reduction)	unoxidized Met + MetSO, II (BrCN method after reduction with $TiCl_3$)	MetSO, III (II − I)	total Met, IV (ion-exchange chromatography)
soybean meal: Brazilian	0.61 (0.61; 0.61)	0.62 (0.62; 0.62)		0.72 (0.71; 0.72)
Brazilian	0.55 (0.54; 0.56)	0.61 (0.60; 0.61)	0.06	0.70 (0.68; 0.71)
U.S.A.	0.43 (0.42; 0.43)	0.44 (0.44; 0.44)		0.55 (0.54; 0.56)
broad beans (*Vicia faba*)	0.19 (0.19; 0.19)	0.19 (0.19; 0.19)		0.22 (0.21; 0.22)
barley	0.16 (0.15; 0.16)	0.17 (0.17; 0.17)		0.20 (0.19; 0.21)
gluten (wheat)	0.79 (0.78; 0.79)	1.10 (1.08; 1.12)	0.31	1.26 (1.24; 1.27)
meat meal tankage	0.80 (0.78; 0.81)	0.80 (0.79; 0.81)		0.79 (0.77; 0.80)
whey powder	0.15 (0.14; 0.16)	0.21 (0.20; 0.21)	0.06	0.20 (0.19; 0.21)
gelatin	0.85 (0.83; 0.87)	0.83 (0.82; 0.83)		
casein (oxidized)	0 (<0.02)	2.57 (2.55; 2.58)	2.57	2.64 (2.61; 2.67)
soy isolate, Promine D	0.18 (0.17; 0.18)	0.98 (0.97; 0.99)	0.80	1.09 (1.07; 1.10)
soy isolate, PP 500 E	1.12 (1.11; 1.12)	1.14 (1.12; 1.16)		1.14 (1.09; 1.19)

[a] In gram(s) per 100 g of product. The results of the two replicate measurements are given in parentheses.

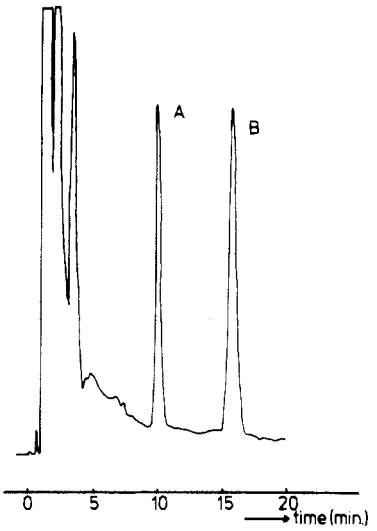


Figure 1. Gas chromatogram of a Brazilian soybean meal, after treatment with cyanogen bromide and titanium trichloride. A = MeSCN. B = EtSCN (=standard).

of Table IV. Some of the values obtained by ion-exchange chromatography are higher than those obtained by the cyanogen bromide/titanium trichloride method, and the reason for this may be the limitations that are inherent with the cyanogen bromide reaction. Some values obtained by ion-exchange chromatography, however, may be rather high, as a result of interfering substances. The procedure developed is very suitable for routine analysis, and up to 60 analyses may be performed in 3 working days. A constraining factor is the time of 20 min for one gas chromatographic analysis. The data presented in Table IV show a substantial amount of methionine sulfoxide in proteins that have been treated with peroxide, e.g., the oxidized casein or the Promine D. Little or not sulfoxide was found in other industrially processed proteins, like soybean meal, meat meal tankage, gelatin, or one of the soy isolates.

Registry No. Methionine, 63-68-3; methionine sulfoxide, 454-41-1; cyanogen bromide, 506-68-3; titanium trichloride, 7705-07-9.

LITERATURE CITED

Block, E. "Reactions of Organosulfur Compounds"; Academic Press: New York, 1978; p 16.
Chasar, D. W. *J. Org. Chem.* **1971**, *36*, 613.
Cuq, J. L.; Besancon, P.; Chartier, L.; Cheftel, C. *Food Chem.* **1978**, *3*, 85.
Ellinger, G. M. *Ann. Nutr. Aliment.* **1978**, *32*, 281.
Ellinger, G. M.; Duncan, A. *Biochem. J.* **1976**, *155*, 615.
Ellinger, G. M.; Smith, R. H. *Biochem. J.* **1971**, *124*, 15P.
Gawargious, Y. A. *Microchem. J.* **1971**, *16*, 673.
Gjøen, A. U.; Njaa, L. R. *Br. J. Nutr.* **1977**, *37*, 93.
Ho, T. L.; Wong, C. M. *Synth. Commun.* **1973a**, *3*, 37.
Ho, T. L.; Wong, C. M. *Synthesis* **1973b**, 206.
Kuzmicky, D. D.; Kohler, G. O.; Walker, H. G.; Mackey, B. E. *Poult. Sci.* **1977**, *56*, 1560.
Lunder, T. L. *Anal. Biochem.* **1972**, *49*, 585.
Neumann, N. P. *Methods Enzymol.* **1967**, *11*, 487–490.
Njaa, L. R. *Br. J. Nutr.* **1980**, *43*, 339.
Sjöberg, L. B.; Boström, S. L. *Br. J. Nutr.* **1977**, *38*, 189.
Slump, P.; Schreuder, H. A. W. *J. Sci. Food Agric.* **1973**, *24*, 657.
Woodham, A. A. In "Nutritional Improvement of Food and Feed Proteins"; Friedman, M., Ed.; Plenum Press: New York, 1978; pp 365–378.

Received for review August 14, 1981. Revised manuscript received July 6, 1982. Accepted September 1, 1982. This investigation was supported by the Produktschap voor Veevoeder, Den Haag.

High-Performance Liquid Chromatographic Determination of Sodium Benzoate When Used as a Tracer To Detect Pulpwash Adulteration of Orange Juice

James F. Fisher

Sodium benzoate was added as a tracer to orange pulpwash. Orange juice diluted with the marked pulpwash was examined by high-performance liquid chromatography for evidence of sodium benzoate. The sample preparation was accomplished with a Sep-PAK C-18 cartridge. Resolution of the sodium benzoate was carried out with a radial compression separation system and an 8-mm C-18 cartridge. Detection was at 230 nm.

Orange pulpwash, a byproduct of the citrus industry, is suspected of being added as a diluent (adulterant) to orange juice. So that this practice can be identified, sodium benzoate, a commonly used food additive and not native to citrus, has been added as a tracer to orange pulpwash.

A practical high-performance liquid chromatographic (HPLC) procedure has been developed to quantitatively identify this marker in both pulpwash and adulterated orange juice. The samples undergo a short preliminary cleanup procedure with final separation of the sodium benzoate by HPLC.

The FDA has approved the use of sodium benzoate at a concentration not to exceed 100 ppm (0.01%) as a tracer in citrus pulpwash when reconstituted to 11.8 °Brix. The Brix value represents the total soluble solids in citrus products [*Natl. Bur. Stand. (U.S.), Circ.*, 1946]. The Florida Citrus Commission has set the lower limit at 50 ppm.

Methods in the literature for the determination of sodium benzoate (Association of Official Analytical Chemists, 1980; Mandrou and Bressolle, 1980; Bennett and Petrus, 1977; Archer, 1980) all entail certain unsatisfactory con-

Florida Department of Citrus, Lake Alfred, Florida 33850.

0021-8561/83/1431-0066$01.50/0