Extraction of Nonprotein Nitrogen from Oilseed Meals with Different Solvents

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ABSTRACT

Nonprotein nitrogen was directly extracted from oil-free meals of rape, sunflower, and soy with seven solvents. Trichloroacetic acid (TCA), sulfosalicylic acid, and acetic acid extracted larger quantities of meal nitrogen than ethanol, chloroform-methanol, and acetone-HCl. Perchloric acid did not extract a significant amount of nitrogen from the meals. However, not all of the nitrogen extracted by TCA (and presumably by sulfosalicylic and acetic acids) was nonprotein nitrogen; it extracted from all the three meals large quantities of protein nitrogen. Ethanol extracts of the meal contained little or no protein nitrogen. Amino acid analyses of TCA and ethanol extracts showed the former to contain three times less total free amino acids than the latter when calculated on extract nitrogen basis but nearly the same, except in soy, when calculated on gram meal basis. TCA and ethanol extracted different quantities of certain amino acids from the same meal. It was concluded that nonprotein nitrogen content of the meals varies with the method and solvent of extraction. Direct extraction of the meal with 80% ethanol is easier, but gives lower yields of basic amino acids lysine and arginine compared with TCA extracts of the same meal.

Although proteins of rape, sunflower, and soy meals have been investigated in some detail (1,2,3) and the amino acid composition of these meals extensively reported (4,5,6,7), few studies have been reported on the nonprotein nitrogen (NPN) content of these or other oilseed meals. The NPN is a complex fraction and contains, in addition to free amino acids and peptides, many nitrogen-containing compounds. Although the NPN may form only a small portion of the total meal nitrogen, its effect on quality and physical appearance of meal and its products may be considerable. The NPN, particularly its amino acid components, may be involved in characteristic flavor of soy meal. In rape meal, NPN is involved in the formation of melanoid compounds which give it a dark brown color (8). The nature and importance of NPN in oilseed meals have yet to be fully investigated.

NPN may be obtained from the meals by two general procedures. It may be directly extracted with a suitable solvent or obtained from aqueous extracts of meals after removing proteins and other macromolecules with precipitants such as trichloroacetic acid (TCA) or sulfosalicylic acid (SSA). The latter procedure is more commonly used but the concentrations of protein precipitants are arbitrarily chosen and may lead to entirely misleading results. For instance, an earlier report (9) showed that a much higher concentration of TCA was required to precipitate proteins from an extract of rape meal than from extracts of sunflower and soy meals.

Becker et al. (10) employed TCA for the direct extraction of NPN from soy meal. Saifer (11) compared a number of organic and inorganic solvents for the extraction of NPN (free amino acids) from human brain tissue and reported

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significant differences in the quantity of certain amino acids in the extracts. For a complete extraction of NPN from peas Bisset (12) suggested hot saline-buffer extraction followed by precipitation of the extract proteins with TCA rather than with ethanol or tungstic acid or by removal of the NPN from the extract by dialysis. On the other hand, Bell (13) extracted wheat flour with water and found dialysis of the extract to give a more reproducible NPN fraction than was given either by heat coagulation of the extract or by TCA precipitation. As far as the authors are aware, no studies have been reported on the extraction of NPN from oilseed meals with different solvents and on the amino acid composition of such extracts.

In the present study, a number of solvents were compared for the direct extraction of NPN from meals of rape, sunflower, and soybean. The solvents, except acetic acid, and their concentrations used were those of Saifer (11). In addition, ethanol and TCA extracts of meal were compared for their free amino acid contents.

MATERIALS AND METHODS

Rapeseed (*Brassica napus* L.), soybean (*Glycine max*), and sunflower (*Helianthus annuus*) were obtained locally from the University increase plots. Sunflower was mechanically dehulled. The oilseeds were defatted by homogenizing twice 5 min. in a Waring Blendor with excess of petroleum ether (Skellysolve F, b.p. 30° to 60° C.). The solvent was removed by filtration and the meals were dried overnight at room temperature. Total nitrogen content of the meals was determined by the micro-Kjeldahl method (14).

Extraction of NPN from the Meals

Solvents used for the extraction of NPN from the meals were: acetic acid (0.05M), ethanol (80% v./v.), chloroform-methanol (1:1), acidified acetone (acetone-HCl), trichloroacetic acid (1% w./v.), sulfosalicylic acid (1% w./v.), and perchloric acid (1 and 6% w./v.). One gram of each meal was shaken in a wrist-arm shaker for 1 hr. at room temperature with 40 ml. of each solvent. The insoluble materials were removed by centrifugation (10,000 × g, 15 min.). The supernatant fraction was made to the original extract volume with distilled water, and an aliquot was taken for the determination of soluble nitrogen (14).

In one experiment the meals were extracted, as described above, with different concentrations of TCA to determine the effect of solvent concentration on nitrogen solubility.

Free Amino Acid Determination of Meal Extracts

Ethanol and TCA extracts of the meals, obtained as above, were used to determine their free amino acid content. The ethanol extract was evaporated to dryness in a rotary evaporator. The residue was dissolved in a small volume of citrate buffer, pH 2.2. The TCA extract was repeatedly shaken in a separatory funnel with water-saturated ether to remove the TCA. The aqueous layer which contained the NPN fraction was evaporated to dryness, as before, and the residue was dissolved in the same buffer. An aliquot of each extract was used for the determination of total nitrogen (14), and for amino acid analysis on a Beckman 120-C amino acid analyzer.

RESULTS AND DISCUSSION

Solubility of Meal Nitrogen in Different Solvents

The quantities of nitrogen extracted by different solvents from the three meals are given in Table I. Rape meal contained more nitrogen soluble in both types of solvents than sunflower and soy meals. This was in agreement with results of a previous study (9) in which the NPN was prepared from these meals by an entirely different procedure. In all the meals TCA and SSA solubilized more nitrogen than the organic solvents. Of the organic solvents, acetic acid extracted the highest quantities of meal nitrogen, followed by ethanol, chloroform-methanol, and acetone-HCl, in that order. Both chloroform-methanol and acetone-HCl extracted less than 1% of the meal nitrogen from sunflower and soy meals and between 1 to 3% of the meal nitrogen from rape meal. Ethanol solubilized two to three times more nitrogen than either of these two solvents. However, ethanol-soluble nitrogen was about one-fourth of that solubilized by acetic acid from rape and soy meals and about one-sixth of that solubilized from sunflower meal. Juo and Stotzky (15) used 0.05M acetic acid for the extraction of basic proteins from a bean seed (*Phaseolus vulgaris* L.). It is therefore likely that acetic acid extracted basic proteins as well as peptide materials from the meals. Rapeseed meal contains a number of basic proteins (1,16) and soy meal has also been reported to contain high quantities of arginine and lysine (3,5). Sunflower proteins have not been well characterized. Like rape and probably other oilseeds, sunflower may contain basic proteins—although this meal is low in lysine—it contains two and one half to three times more arginine than lysine (5,6,7).

TCA was the most effective solvent and extracted 29% of the meal nitrogen from rape meal and between 12 and 15% of the meal nitrogen from the other two meals (Table I). SSA solubilized somewhat lower quantities of nitrogen from rape and sunflower meals but much lower (almost half of that solubilized by TCA) from soy meal. Perchloric acid used at 1 or 6% failed to solubilize a significant amount of nitrogen from the meals. This was surprising since Saifer (11) reported that perchloric acid extracted the same quantities and types of amino acids from human brain tissue as did TCA and SSA. It is difficult to give a satisfactory explanation for the lack of solubility of oilseed meals nitrogen in this solvent. Not all the nitrogen solubilized by 1% TCA was NPN. Amino acid analysis of TCA and ethanol extracts showed that the TCA extract contained

TABLE I. NITROGEN CONTENT OF RAPE, SUNFLOWER, AND SOY MEAL EXTRACTS OF DIFFERENT SOLVENTS[a]

Solvent	Rape	Sunflower	Soy
	% of meal nitrogen		
Trichloroacetic acid (1%)	29.0	15.4	12.3
Sulfosalicylic acid (1%)	22.0	14.9	5.6
Perchloric acid (1 and 6%)	0.0	0.0	0.0
Acetic acid (0.05M)	17.1	14.5	5.8
Ethanol (80% v./v.)	4.4	2.3	1.4
Chloroform-methanol (1:1)	2.7	0.9	0.6
Acetone-HCl	1.5	0.7	0.7

[a]Average of duplicate determinations.

one-third the concentration of free amino acids found in the ethanol extract, although TCA extracted three times more nitrogen from rape and sunflower meals and two times more from soy meal.

	Rape		Sunflower		Soy	
	TCA	Ethanol	TCA	Ethanol	TCA	Ethanol
Nitrogen per ml. extract (mg.)[a]	1.71	0.45	1.03	0.35	0.41	0.27
Total free amino acids (μmoles per mg. extract nitrogen)[b]	2.84	10.52	3.69	10.15	6.01	16.64

[a]Total volume 10 ml. from 2 g. of meal.

[b]Excludes serine and threonine which were not separated.

TCA and presumably SSA and acetic acid extracted proteins as well as NPN from the meals. During extraction of the meals with 1% TCA the pH of extracts of rape, soy, and sunflower rose from an initial value of 1.40 to 2.05, 2.45, and 2.60, respectively. Therefore, the change in hydrogen ion concentration of the solvent was sixfold in rape, tenfold in soy, and twelvefold in sunflower. Under these conditions the formation of a weak salt may also be partly responsible for the solubilization of meal proteins. To determine the quantity of protein extracted by TCA, an aliquot of 1% TCA extract was adjusted to a final TCA concentration of 14.5% (w./v.) and the proteins precipitated were removed by centrifugation (10,000 × g, 10 min.). Data in Table II show that 78% of nitrogen present in rape meal extract, 51% in sunflower extract, and 24% in soy extract was precipitated as protein nitrogen. Similar treatment of 80% ethanol extracts of the meals with 14.5% TCA precipitated only 5% protein nitrogen from rape meal extract, less than 2% from sunflower extract and none at all from soy extract.

TCA Concentration and Nitrogen Solubility

Effect of 0.5 to 20% TCA on nitrogen solubility of the meals is shown in Fig. 1. A TCA concentration of 1% solubilized the most nitrogen from rape and sunflower meals, but with soy meal 0.5% TCA was most effective. Extractions of the meals with TCA concentrations up to 20% did not cause any protein hydrolysis, as shown by decreasing solubility of meal nitrogen. Only a 50% TCA concentration caused severe hydrolysis of the meal proteins (not shown in Fig. 1). The solubility data show a typical "salting out" effect with an increase in TCA concentration. This effect varied among the meals and was probably caused by

TABLE II. PERCENT NITROGEN PRECIPITATED BY 14.5% TCA FROM 1% TCA AND 80% ETHANOL EXTRACTS OF RAPE, SUNFLOWER, AND SOY MEALS

Meal	1% TCA Extract	80% Ethanol Extract
Rape	78.0	5.0
Sunflower	51.2	1.7
Soy	24.4	0.0

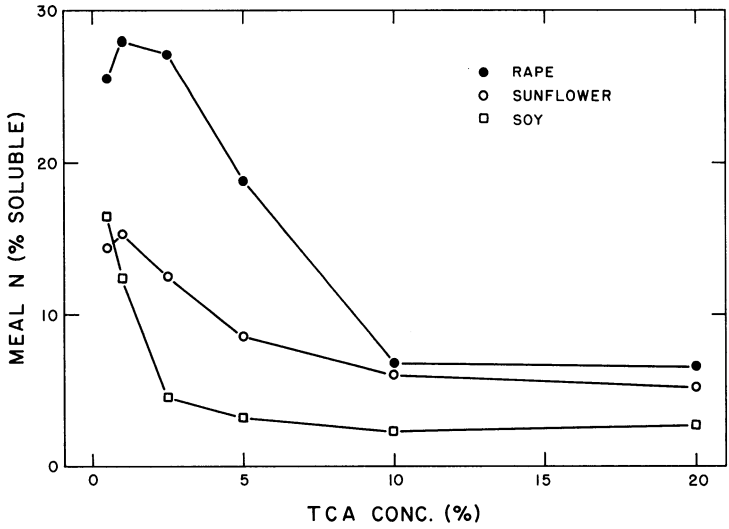

Fig. 1. The effects of different concentrations of trichloroacetic acid on the solubility of nitrogen compounds extracted from rape, sunflower and soy meals.

number and size of proteins present in the meals. For instance, in rape meal an increase in TCA concentration from 2.5 to 5.0% decreased nitrogen solubility by 9%. A further twofold increase in the solvent concentration decreased solubility by 12%. In sunflower meal the corresponding figures were about 4 and 2.5%. In soy meal the decrease in solubility at these concentrations of TCA was minimal (1%). In this meal maximum decrease (8%) in solubility occurred at a TCA concentration of 2.5%.

Becker et al. (10) reported solubility of soy meal nitrogen in concentrations of TCA ranging from 0.2N (3.3%) to 4.0N (65.4%). They selected a TCA concentration of 0.8N (about 13%) from a range of 0.65 to 1.0N (10.6 to 16.3%) for the extraction of NPN from soy meal because of little change in protein solubility in this range. In the present study also, protein solubility in the three meals was minimal at TCA concentrations of 10 and 20% (Fig. 1). The quantities of NPN solubilized at these concentrations of TCA were considerably higher than those solubilized by 80% ethanol (see Table I), a common solvent for the extraction of NPN from plant materials (17). TCA and ethanol may, however, extract different kinds of amino acids from the meals. Saifer (11) has reported that ethanol, like other organic solvents, extracted from the brain tissue lower quantities of basic amino acids compared to perchloric acid (and TCA). Higher values of NPN obtained with 10 and 20% TCA than with 80% ethanol may be due to different amino acid content of these extracts.

Free Amino Acid Composition of TCA and Ethanol Extracts

Rape meal contained more total free amino acids soluble in TCA or ethanol than sunflower and soy meals (Table III). Except in the case of sunflower meal, TCA and ethanol extracted almost the same quantities of total free amino acids. The three meals contained in a free state all the common protein amino acids

TABLE III. FREE AMINO ACID CONTENTS OF TCA AND ETHANOL EXTRACTS OF RAPE, SUNFLOWER, AND SOY MEALS (AVERAGE OF DUPLICATE DETERMINATIONS)

Amino Acid	Rape		Sunflower		Soy	
	TCA	Ethanol	TCA	Ethanol	TCA	Ethanol
	(μmoles per g. oil-free meal)					
Tryptophan	0.11	0.16	0.87	2.06	1.26	1.36
Lysine	0.38	0.03	0.22	0.09	0.43	0.31
Histidine	0.07	0.37	0.09	0.12	0.26	0.24
Ammonia	3.50	2.38	1.15	3.76	3.78	3.26
Arginine	0.46	0.10	3.07	1.39	1.90	1.47
Aspartic acid	3.81	2.46	1.88	4.65	2.21	2.15
Threonine	...	...	...	...	...	...
Serine	...	...	...	...	...	...
Glutamic acid	14.61	16.67	1.43	3.35	2.49	3.34
Proline	0.27	0.35	0.16	0.49	0.34	0.55
Glycine	0.15	0.13	0.19	0.38	1.23	0.88
Alanine	0.34	0.50	0.73	1.93	2.46	2.79
Cystine	0.00	0.00	0.00	0.00	0.00	0.00
Valine	0.19	0.19	1.28	3.10	0.39	0.59
Methionine	0.03	0.03	0.04	0.10	0.09	0.11
Isoleucine	0.03	0.04	0.08	0.22	0.28	0.45
Leucine	0.02	0.04	0.12	0.27	0.10	0.16
Tyrosine	0.08	0.11	0.02	0.09	0.14	0.18
Phenylalanine	0.29	0.14	0.19	0.46	0.35	0.26
Total	24.34	23.71	12.51	22.46	17.73	18.12

except cystine and methionine, which were present in small quantities in TCA as well as in ethanol extracts of the meals. Threonine and serine were poorly separated because of the interference of unknown compounds and hence could not be estimated. Both TCA and ethanol extracts contained compounds, presumably peptides, which were eluted before aspartic acid. The presence of these compounds did not affect the resolution of amino acids eluted after aspartic acid, since retention times of the protein amino acids are accurately known. No attempts were made to identify these and other unknown amino acids present in both the extracts.

The major free amino acids of TCA and ethanol extracts of rapeseed were glutamic and aspartic acids. These two amino acids and ammonia formed 80% of the total free amino acids in this meal. The other amino acids were present less than 0.5 μmoles per g. meal, though there were substantial differences in the quantities of lysine, histidine, arginine and phenylalanine in TCA and ethanol extracts of rape meal. The free amino acid composition of sunflower and soy meals was different than that of rape meal. These meals contained much lower quantities of glutamic and aspartic acids but higher quantities of ammonia (except TCA extract of soy meal), tryptophan, lysine (only sunflower), arginine, glycine, alanine, and valine than rape meal. There were also differences in free amino acid composition of sunflower and soy meals. Sunflower contained more lysine, tryptophan (only TCA extract), histidine, glutamic acid, proline glycine, alanine, isoleucine, and tyrosine but less of arginine, aspartic acid (ethanol extract) and valine.

In all three meals TCA and ethanol extracted different quantities of certain

amino acids. For example, TCA extracted more lysine and arginine but less aspartic acid (especially in soy) glutamic acid, proline, alanine and valine (except in rape) than ethanol. These results tend to agree with the findings of Saifer (11) that ethanol and other organic solvents give low yields of basic amino acids as compared to perchloric acid.

CONCLUSIONS

The results show that direct extraction of rapeseed, sunflower, or soy meals with various solvents gives different amounts of NPN compared with extraction of the meals with dilute alkali and subsequent TCA precipitation. Protein precipitants such as TCA or SSA and acetic acid used at a low concentration extracted substantial amounts of meal proteins. Concentrations of these solvents at which protein solubility is minimal may be employed for the extraction of NPN as has been done with TCA for soybean meal (10). This optimum concentration, however, needs to be determined for each species and cultivar because of the variations in the solubilities of the nitrogen-containing compounds. Furthermore, removal of TCA from meal extract is tedious. Repeated washings with water-saturated ether are required and may cause a loss of NPN. Direct extraction of meal with 80% ethanol is easier but this method may give low yields of basic amino acids particularly lysine and arginine. These results and previous ones (9) have shown that the NPN content (and consequently, the free amino acid content) depends upon the method and solvent of extraction. Similar variations have been found in experiments to find the free amino acid content of mammalian tissue (11). Thus the results of the present work give support to the idea that the free amino acid content of a seed should not be used for its identification or to distinguish between cultivars or species.

Literature Cited

1. BHATTY, R. S., MacKENZIE, S. L., and FINLAYSON, A. J. The proteins of rapeseed *(Brassica napus* L.) soluble in salt solutions. Can. J. Biochem. 46: 1191 (1968).
2. JOUBERT, F. J. Sunflower seed proteins. Biochim. Biophys. Acta. 16: 520 (1955).
3. WOLF, W. J. Soybean Proteins: Their functional, chemical, and physical properties. J. Agr. Food Chem. 18: 969 (1970).
4. MILLER, R. W., Van ETTEN, C. H., McGREW, C., WOLFF, I. A., and JONES, Q. Amino acid composition of seed meals from forty-one species of Cruciferae. J. Agr. Food Chem. 10: 426 (1962).
5. EVANS, R. J., and BANDEMER, S.L. Nutritive values of some oilseed proteins. Cereal Chem. 44: 417 (1967).
6. GHEYASUDDIN, S., CATER, C. M., and MATTIL, K. F. Preparation of a colorless sunflower protein isolate. Food Technol. 24: 36 (1970).
7. TKACHUK, R., and IRVINE, G. N. Amino acid compositions of cereals and oilseed meals. Cereal Chem. 46: 206 (1969).
8. RUTKOWSKI, A. Effect of processing on the chemical composition of rapeseed meal. Proc. Int. Rapeseed Conf., Ste. Adele, Quebec 496 (1970).
9. BHATTY, R. S. A note on trichloroacetic acid precipitation of oilseed proteins. Cereal Chem. 49: 729 (1972).
10. BECKER, H. C., MILNER, R. T., and NAGEL, R. H. A method for the determination of non-protein nitrogen in soybean meal. Cereal Chem. 17: 447 (1940).
11. SAIFER, A. Comparative study of various extraction methods for the quantitative determination of free amino acids from brain tissue. Anal. Biochem. 40: 412 (1971).
12. BISSET, S. K. The non-protein nitrogen of extracts of *Pisum sativum.* Biochem. J. 58: 225 (1954).
13. BELL, P. M. Non-protein nitrogen in wheat flour. J. Sci. Food Agr. 14: 133 (1963).

14. ASSOCIATION OF ANALYTICAL CHEMISTS. Methods of analysis (11th ed.), p. 858. The Association: Washington, D.C. (1970).
15. JUO, P.-S., and STOTZKY, G. Changes in protein spectra of bean seed during germination. Can. J. Bot. 48: 1347 (1970).
16. SWANLJUNG, P. Purification and some properties of a group of small basic proteins from rapeseed. Acta Chem. Scand. 26: 405 (1972).
17. STEWARD, F. C., and DURZAN, D. J. Metabolism of nitrogenous compounds. In: Plant Physiology: A Treatise, IVA, ed. by F. C. Steward. Academic Press: New York (1965).

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