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EFFECT OF SOLVENTS ON THE EXTRACTABILITY OF LIPIDS FROM LEAF PROTEINS

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The effect of solvents on the extractability of lipids of leaf protein concentrates and their composition was studied. Extraction with acetone removed most of the chlorophyll, triglycerides and some phosphatides. The mixtures of acetone--water extracted more lipids as compared with pure acetone. Treatment of leaf protein concentrate with acetone before extraction with chloroform--methanol resulted in a decrease in the total amount of lipid phosphorus and lipid nitrogen. The best extraction of lipids was obtained with 2:1 mixture of chloroform--methanol.

Plant lipids are difficult to isolate and subsequently, purify, because they are present as complexes with carbohydrate, proteins and chlorophyll.[1–5] In order to release the lipids from these complexes, it is necessary to employ such denaturing agents as methanol, ethanol or acetone, which rupture the linkages between the lipids and other constituents. As many of the commonly occuring lipids are not soluble in these solvents, a nonpolar solvent, such as petroleum ether, chloroform or diethylether, is usually added to assist the extraction.

Ethanol or an ethanol–ether mixture is frequently used for liberating bound lipids from wet tissues.[6] Bloor[7–8] used as 3:1 ethanol–ether mixture for the extraction of blood lipids; Hanahan and Chaikoff[9] used the same mixture at 55–60°C for removing lipids from cabbage leaves. With animal tissues Folch, Lees and Sloane-Stanley[10] used a 2:1 mixture of $CHCl_3$–CH_3OH but Bligh and Dyer[11] preferred 2:2:1 $CHCl_3$–CH_3OH –H_2O when extracting fish tissues; Thuille and Loizeau[12] used 1:2:4 methanol–chloroform–water for extracting egg yolks. Lima *et al.*[13] and Buchanan[14] used chloroform–methanol mixture (2:1 v/v) for extraction of freeze-dried leaf proteins.

Acetone, like alcohol, possesses considerable lipid-freeing power[15] (cf. Lester and Fleischer[16]), but being a poor solvent for phosphatides cannot be used for the complete removal of lipids. Extraction by acetone is generally followed by a solvent such as chloroform.

Since most of the lipids present in plants resemble those in animal tissues, therefore, the same methods of extraction can be used. This report studies the recovery and nature of the lipids extracted from wheat and Kale proteins by various solvents.

Materials and Methods

Preparation of Proteins.—The proteins extracted from kale (*Brassica cleracea*), wheat (*Triticum vulgare*) and maize (*Zea mays*) leaves, by the method of Morrison and Pirie,[17] were used for comparative studies of the methods of extraction. The proteins were washed with water, filtered and pressed to remove excess water. The pH of wheat, maize and kale proteins were 6.25, 6.9 and 6.4 respectively.

Five-hundred-gram lots of wheat, maize and kale proteins were freeze-dried.

Acetone Extraction.—(a) Two-gram samples of freeze-dried wheat protein were soaked in 40 ml portions of acetone–water mixtures of varying ratio for 1 and 3 hr. The protein suspensions were transferred to Buchner funnels and the acetone–water soluble lipids removed by suction.

(b) Five grams of wheat protein cake (dry matter (D.M. 39.8%) was extracted with 100 ml of hot acetone (Soxhlet) for 6 hr followed by extraction with hot chloroform for another 4 hr. The residue was finally extracted with 2:1 $CHCl_3$–CH_3OH mixture at room temperature.

(c) Five grams of wheat protein-cake was extracted three times with 50 ml of acetone at room temperature; the residue was extracted three times with 50 ml 2:1 $CHCl_3$–CH_3OH mixture.

(d) A mixture of proteins extracted from tare (*Vicia sativa*), tomato (*Lycopersicum esculantum*) and barley (*Hordeum vulgare*) leaves was examined. 16 9 kg protein (D.M. 33 8% were soaked in 34 of acetone for 18 hr. The acetone extract which contained lipids, water and other substances was drained off. The remaining solids were then extracted with six portions of 30 ml of acetone, each time for 2 hr.

The lipids in each extract were analysed.

Other Methods of Lipid Extraction.—The lipids present in the kale, maize and wheat proteins were also extracted by the methods of Hanahan and Chaikoff,[9] Folch *et al.*[10] Bligh and Dyer,[11] and Thuille and Loizeau.[12]

The methods of Bligh and Dyer[11] and Thuille and Loizeau[12] were followed without alteration. But the extraction by Hanahan and Chaikoff[9] method was done at room temperature instead of at 55–60°C. The method of Folch *et al.* was slightly modified. The details are given below:

Two grams freeze-dried protein was soaked for 1 hr in sufficient water to bring the moisture content

to about 80% and then extracted three times with a five-fold volume of 2:1 $CHCl_3$–CH_3OH at room temperature. The extracts were combined, shaken with water (the volume of water, including that added in the beginning, was 1/5th of the volume of $CHCl_3$–CH_3OH used), and allowed to separate; the upper (methanol–water) and lower (chloroform layers contained the water soluble substances and lipids respectively.

Analysis

Dry Matter.—The dry matter contents of leaf protein preparations were determined by drying the samples at 100°C for 40 hr. Lipids extracts were concentrated at 40°C under reduced pressure and dried (H_2SO_4).

Nitrogen.—Nitrogen was determined by a microkjeldahl method using K_2SO_4–$CuSO_4$–SeO_2 (9:1:0.02) catalyst.

Phosphorus.—Total phosphorus was determined by the method of Holden and Pirie and nucleic acid–phosphorus by the method of Spirin.

Chlorophyll.—The chlorophyll contents of the protein samples were determined by the method of Arnon (using filter 607 in an EEL colorimeter.

Results and Discussion

Extraction of Lipids with Acetone

Method (*a*).—Freeze dried wheat protein when soaked in acetone for 1 hr released about 70 mg of lipids/g of protein (Fig. 1). No more lipids were extracted when the time of soaking was increased 3 hr. Addition of water to the acetone increased the amount of lipids extracted; Fig. 1 shows that most lipids are extracted when the solvent mixture is 9:1 acetone–water. When the experiments were repeated with acetone containing between 1 and 10% of water it was found that a mixture containing 8% of water extracted most nitrogen, chlorophyll and lipids. Most phosphorus was, however, extracted when solvent contained 10% water (Fig. 2) (cf. Lester and Fleischer[16])

These results indicate that the amount of water present in the tissue affects the amount and composition of the lipids extracted by acetone when it is used either as a dehydrating agent or for breaking lipid–protein or lipid–carbohydrate complexes.

Method (*b*).—The hot Soxhlet extraction of wheat protein with acetone for 4 hr released more lipids, and more nitrogen and phosphorus, than the cold extraction procedure. The residue when extracted with hot $CHCl_3$ (Soxhlet) for 4 hr released 21 mg of lipid with a N–P ratio 1:0.83 (Table 1). Further extraction of the residue with 2:1 $CHCl_3$–CH_3OH (at room temperature) recovered 17 mg of lipids. The total amount of

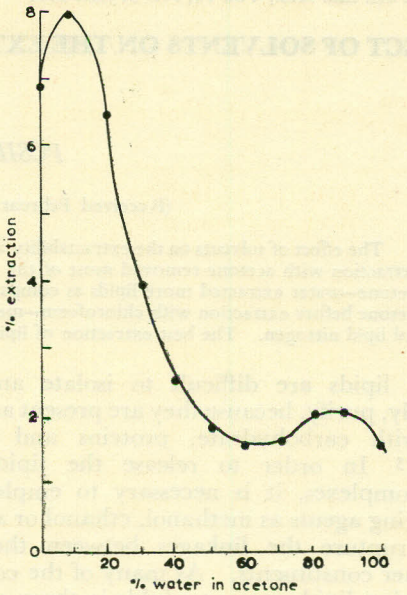

Fig. 1.—Effect of addition of water to acetone on the extractability of lipids. Solvent-protein ratio 20:1. Time of soaking 1 hr.

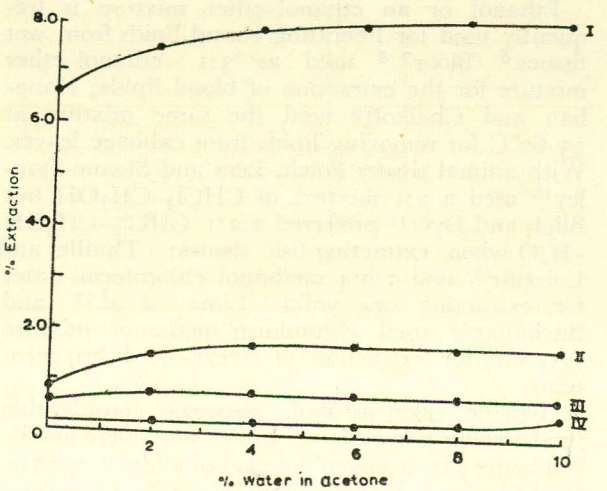

Fig. 2.—Effect of concentration of water in water–acetone mixture on the extractability and composition of lipids. (i) lipids extracted g/100g, (ii) nitrogen mg/g, (iii) chlorophyll g/100g, (iv) phosphorus mg/g.

lipids extracted by these methods and the composition of the extracted lipids was almost the same.

Method (*c*).—Wheat protein cake when dehydrated by extracting three times with acetone, at room temperature, released 183 mg of lipid/g of protein, which contained almost all the chlorophyll, 1.59 mg of nitrogen and 0.29 mg of phosphorus. Further extraction of the residue with with 2:1 $CHCl_3$–CH_3OH mixture released 85 mg

TABLE 1.—EFFECT OF ACETONE PRETREATMENT ON EXTRACTABILITY AND COMPOSITION OF WHEAT PROTEIN (D.M. 39.8%).

Extraction sequence	Solvents used for extraction	Extractable lipid	mg/g of protein		
			Lipid-N	Lipid-P	Lipid-Chl
	$CHCl_3$–CH_3OH (2:1) only	252	3.20	2.18	11.9
1	Acetone (room temp)	183	1.59	0.29	11.4
2	$CHCl_3$–CH_3OH (2:1) (room temp)	85	0.80	1.21	—
1	Acetone hot	229	2.04	0.76	11.3
2	$CHCl_3$ hot	21	0.18	0.34	—
3	$CHCl_3$–CH_3OH (room temp)	17	0.41	0.31	—

TABLE 2.—COMPARATIVE STUDY OF EXTRACTION OF LIPIDS FROM WHEAT, KALE AND MAIZE PROTEINS.

Solvent used for extraction	Wheat protein mg/g of protein				Kale protein mg/g of protein				Maize protein mg/g of protein			
	Extractable matter	N	P	Chl	Extractable matter	N	P	Chl	Extractable matter	N	P	Chl
$CHCl_3$--CH_3OH (2:1)[a]	252	3.2	2.18	12.2	232	2.94	3.5	14.5	239	2.97	2.89	12.0
$CHCl_3$--CH_3OH--H_2O (2:2:1)[b]	243	2.56	1.84	12.0	204	2.11	3.38	14.0	222	2.67	2.43	12.8
$CHCl_3$--CH_3OH--H_2O (2:1:4)[c]	197	1.8	1.68	11.6	206	2.19	3.02	12.8	203	1.95	2.01	11.7
C_2H_5OH--$(C_2H_5)_2O$ (3:1)[d]	207	2.07	1.24	8.2	195	1.69	2.95	11.2	202	1.84	1.23	8.0

(*a*) F. Folch M. Lees and G.H. Sloane-Stanley, J. Biol. Chem., **26**, 497 (1937); (*b*) E.G. Bligh,s and W. J. Dyer, Can. J. Biochem. Physiol., **37**, 911 (1959); (*c*) M. J. Thuille and M.F. Loizeau, Ann. Biol. animale Biochem. Bophys 1., 98 (1961); (*d*) D. J. Hanahan and I.L. Chaikoff, J. Biol. Chem., **72**, 191 (1948).

TABLE 3.—COMPOSITION OF LIPIDS EXTRACTED WITH ACETONE FROM A MIXTURE OF TARE, BARLEY AND TOMATO LEAVES PROTEIN.

Fraction	Soaking time hr	mg/g of protein			
		Extractable lipid	Lipid-N	Lipid-P	Lipid-Chl
1	18	56 7	0 6	0 24	3.62
2	2	59 0	0 52	0 21	3.58
3	2	25 8	0 20	0 10	1.53
4	2	7 0	0 04	0 04	0 32
5	2	2 8	0 02	0 02	0 09
6	2	1 05	0 01	trace	0 03
7	2	0 55	0 01	trace	0 01

of lipid which contained 0 80 mg of nitrogen and 1.21 mg of phosphorus (Table 1).

Method (*d*).—The results of large scale extraction of a mixture of tares, tomato and barley proteins with acetone show that the extract contains most of the chlorophyll present in the protein and small amounts of nitrogen and phosphorus (Table 3). About 97% of the acetone–soluble lipids were extracted in the first four extractions. Henry (Private communication) observed that the digestibility of the protein increases significantly after extraction with acetone. This seems to result from the breaking of the linkages between lipids and protein by acetone.

These results when compared with those from the direct extraction with 2:1 $CHCl_3$–CH_3OH (at room temperature) show a 31–35% decrease in the amount of lipid–phosphorus and a 18–25% decrease in lipid–nitrogen. The smaller nitrogen content appears to be due to the breaking of the linkages between the lipids and protein by acetone. Le Baron and Lees[18] made similar observations on the lipids extracted from brain tissues.

Other Methods.—A comparative study of four methods of extraction of lipids from kale, maize and wheat proteins (Table 2) shows that the lipids extracted by the slightly modified method of Folch *et al.* contain the highest amounts of nitrogen and phosphorus; more lipids per gram of protein were also recovered. The results were reproducible and there was no difficulty in separating the lipids and non-lipids fractions.

For this reason the method of Folch *et al.* is recommended for the extraction of lipids from leaf protein.

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