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13. ABSTRACT (MAX 200 WORDS): UCHON ET AL PRESENTED A METHOD BASED ON TAKA-DISTASE HYDROLYSIS REPLACING THE HYDROCHLORIC ACID HYDROLYSIS. THIS WORK DESCRIBES REFINEMENT OF THIS METHOD WHICH IS NOW USED FOR FAT DETERMINATION IN NUTRITIONAL ASESSMENT OF MILITARY RATIONS.THE METHOD		
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ENZYMATIC HYDROLYSIS AND SOLVENT EXTRACTION METHOD OF FAT DETERMINATION

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INTRODUCTION

Fats are recognised as important dietary components, with water, protein and carbohydrate. Fat has particular significance because it yields twice the energy compared with the other macro-nutrients. Military rations are designed to provide military personnel with adequate nutrition in as compact and light weight manner as possible. Military activities require high levels of energy expenditure. Current nutritional wisdom is that energy derived from fat should not exceed 33% of total energy intake [1]. Furthermore, polyunsaturated fatty acids are essential in animal nutrition, particularly human nutrition with a recommended intake of 1.5 to 8% of energy [2]. Thus, there is need for a consistent method for determination of fat in foods.

Recently, a collaborative study on fat in cereal products using the currently recognised methods found significant discrepancies between recognised methods [3]. It also found that precision was poor with variations of 10 to 50% RSD. The introduction of nutrient labelling has made this a matter for urgent resolution.

Fat has been determined in foods by a number of different methods of analysis. One reference [4] includes 29 variations of extraction methods for determination of fat. Lichon et al [5] discussed the problems with some of these methods. A particular concern was that the hydrochloric acid hydrolysis method would convert sugars to furfurals, which would polymerise to produce ether extractables. These extractables would be determined as fat. Lichon et al [5] presented a method based on taka-diastase hydrolysis replacing the hydrochloric acid hydrolysis.

This work describes refinement of the method [5], which is now used for fat determination in nutritional assessment of military rations.

METHOD

Weigh 1g sample accurately into Mojonnier flask. Include a blank in each run for each enzyme solution used, and at least one duplicate. Add a small quantity of water (ca 2 mL) to dry foods and immediately disperse the sample to a slurry at 45°C in water bath, breaking up any lumps. Add 10 ml enzyme dispersion of either, 10g taka-diastase (Serva 35740) and 2g papain (BDH 39030) in 200 mL 0.5N phosphate buffer for most mixed foods, or 2g Proteinase A (Serva 31439) in 200 mL pH 8.0 borax/HCl buffer, for very high protein foods (eg meat, cheese) and immediately disperse with the aid of

heating at 45°C in a water bath. Incubate full set of samples with agitation using an orbital incubator at 37°C overnight. Cool and add 10 mL ethanol and bring up to bottom neck of flask with water add 25 mL diethyl ether, shake and allow to settle. Add 25 mL petroleum ether, shake and allow to settle. Pour upper layer into 100 mL round bottom flask. Commence evaporation of first extract with rotary evaporator under vacuum at 40 - 70°C to a small volume using a five flask manifold. A slow nitrogen gas bleed will prevent bumping and minimise oxidation of fat when further analysis for fatty acids is required. The ethers are collected with the aid of condensers cooled with a refrigerated coolant of 1 methanol: 9 water to minimise loss of diethyl ether. Extract twice more with 30 mL recovered mixed ethers (1 diethyl ether: 1 petroleum ether, sg 20°C 0.692) evaporating down at 40 - 70°C after each extraction in the same flask. If phospho-lipids and other more polar lipids are to be extracted, a further two extractions with 30 mL chloroform will be necessary. Evaporate the chloroform extracts down in the same flask at 30 - 50°C. Evaporate final extract to apparent dryness. Remove from rotary evaporator and while fat is still warm, blow a fine jet of nitrogen gas over surface of the extract, agitating to remove residual solvent in a fume cupboard. Dry extracts either; under vacuum at room temperature for at least 3 hours and break vacuum with nitrogen to prevent oxidation, if the fat is to be further analysed for fatty acids; or dry extract at 100°C for 1 hour, removing from oven after first 15 minutes to blow a fine jet of nitrogen gas over the surface of the extract and agitating to remove residual solvent in a fume cupboard. Repeat drying step until constant weight is achieved. Wash out flasks with petroleum ether until cleaned of fatty matter. Place vacuum dried fat extract in vial and allow mixed ethers to evaporate and store under nitrogen in freezer in sealed vials until fatty acids can be determined. Redry the flask as before, reweigh and calculate proportion of fat in sample from the difference correcting for any blank.

RESULTS AND DISCUSSION

Lichon et al [5] found that the enzymatic method was applicable to a range of foods and showed that it obtained a zero value for fat in sugar. Thus, the method overcame the problem of furfural formation but still provided for the hydrolysis of starch and proteins.

Care must be taken to properly disperse dry samples in water by preparing samples individually according to the method. Similarly samples must be thoroughly dispersed after adding the enzyme. Incomplete dispersion will lead to problems achieving complete hydrolysis and repeatable results.

Further confirmation has been obtained through participation in the NATA biscuit proficiency program [6] involving over 40 laboratories with the results shown in Table 1. The enzymatic results were within one standard deviation of the pooled results for all methods. The Mojonnier method (acid hydrolysis) is the official method for these products hydrolysing and releasing bound fat, but the milk arrowroot biscuits are sweetened and would also provide sugar for furfural formation. The enzymatic results are slightly lower than the Mojonnier ones but higher than the Soxhlet results, suggesting that it does provide more reliable results.

Some problems were detected using this method with mixed solvents and using it with high protein foods (Table 2). It is important to add the first 25 mL diethyl ether and mix it with the hydrolysate followed by 25 mL petroleum ether. If the two solvents are added together or the proportion of diethyl ether falls, such as may happen during recovery of solvents,

then the fat result is depressed. Provided the proportions of the ethers remain equal, the second and third extractions may be done with the mixed ethers, allowing the use of recovered solvent.

Table 1: Comparison of Fat results in biscuits [6]

Method	Milk Arrowroot A	B	Sao C	D
All Methods Pooled	10.9±0.6	10.6±0.8	15.5±1.0	14.7±0.9
Mojonnier	11.3±0.4	11.0±0.3	16.5±0.7	15.2±0.7
Soxhlet	10.5±0.3	10.0±0.4	14.8±0.9	13.8±0.5
Enzymatic (Taka/papain)	10.3 10.7	11.0 10.7	16.0 16.1	15.1 15.2

Table 2: Fat in liquid milk by AS2300 [7] and taka-diastase/papain

Method	Sample	Percent Fat Mean	σ	N	
AS2300 adding diethyl either separate from petroleum ether 1st extract 1:9 2nd/3rd	A	25.6	0.2	4	
	B	24.0	2.0	4	
AS2300 Mixed 1:9 solvents	A	1.3		0.7	4
	B	2.0		1.7	4
AS2300 Separate additions all extractions	A	26.1	0.6	4	
	B	26.2	0.4	4	
AS2300 Separate additions 1st extract 1:1 2nd/3rd	A	26.1	0.3	4	
	B	26.3	0.3	4	
Taka-diastase/Papain hydrolysis 37° 16 h	A	25.6	0.5	4	
	B	25.5	0.5	4	
Taka-diastase/Papain hydrolysis 45° 2 h	A	24.8	0.7	4	
	B	24.7	0.5	4	
Interlaboratory AS1629*	A	26.5	0.5	45	
	B	27.0	0.6	45	

* NATA [8], result with outliers removed.

The taka-diastase/papain enzyme mixture has not been able to fully recover fat from high protein foods such as liquid milk as compared with alkaline ammonia hydrolysis [8].

The enzymes pepsin, protease A and protease B were evaluated for hydrolysis of high protein foods. Protease A, at pH 8, was chosen because on sweetened condensed milk it gave a fat result (9.04%), close to that obtained with the official method (9.14%) [6], while the pepsin was similar (9.00%), but protease B was much lower (7.89%). The hydrolysate produced with protease A was translucent and very similar to that obtained from an ammonia hydrolysis [7]. The protease A hydrolysis was used in a meat and bone meal proficiency test of over 40 laboratories [9] giving results (12.0%, 12.3%) within one standard deviation of the mean (11.7 ± 0.6%).

The phospholipid fraction and other more polar lipids can be included with the addition of an extraction step using chloroform. When this fraction has been measured separately it has been a very small proportion of the total (0.1 to 0.4%), which is of the order of the error for this method.

Vacuum drying of the residue and evaporation of solvent with the aid of nitrogen, rather than air, provides the analyst with a fat extract suitable for derivatising to the fatty acid methyl esters and determining fatty acid profile. When the profiles obtained from the extracts from samples of ham, including chloroform extracts, were compared there were no significant differences in twenty two fatty acids, except that the taka-diastase/papain extract was nearly two standard deviations lower for palmitic acid (20.6%, 24.3 ± 2.1%) but higher for linoleic acid (12.0%, 8.1 ± 2.4%).

CONCLUSIONS

The first extraction with diethyl ether and petroleum ether is critical to reliable recovery of the fat present in the ammonia hydrolysis method, the hydrochloric acid hydrolysis method and the taka-diastase hydrolysis method.

The taka-diastase method for fat gives low results with dairy products, which are considered to be due to inefficient hydrolysis of the lipo-protein fraction by the enzymes. High protein foods should therefore be hydrolysed with protease A enzyme.

The method avoids the problems of furfural formation from the use of acid hydrolysis, while giving comparable results to the recognised methods. It is possible to undertake the determination in runs of up to twenty determinations.

It is suggested that the method would be suitable as a general purpose method for use in nutrient labelling.

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