JOURNAL
OF THE
SCIENCE OF FOOD AND AGRICULTURE
(INCLUDING ABSTRACTS)

Published by the Society of Chemical Industry

Volume 16 | No. 11 | November, 1965

SOCIETY OF CHEMICAL INDUSTRY

FOUNDED IN 1881 INCORPORATED BY ROYAL CHARTER 1907

Offices of the Society: 14 Belgrave Square, S.W.1
Telephone: BELgravia 3681/5

Annual Subscription to the *Journal of the Science of Food and Agriculture*
£15 post free, single copies £1 17s. 6d. post free

THE DETERMINATION OF NITROGEN IN WOODY TISSUE

By P. J. RENNIE

A simple routine semi-micro Kjeldahl procedure is described suitable for woody tissues. Some 250 mg. of milled tissue is digested with potassium sulphate, conc. sulphuric acid and mercuric oxide under a thermostatically controlled three-stage electrical heating schedule, digestion being continued for 2 h. after clearing.

Tests conducted with a variety of woody tissues give good reproducibility—coefficients of variation being all $<3{\cdot}5\%$, and N-content values higher than by three other procedures based on combinations of selenium, copper sulphate and vanadium pentoxide. Recoveries from a range of N-reference standards typical of compounds or structures present in plant materials show, with two exceptions, a high accuracy—within $\pm 0{\cdot}6\%$, and a high precision—coefficients of variation $<0{\cdot}6\%$

The poor recovery ($85{\cdot}3\%$) obtained with canavanine, which contains a N–O bond, is discussed and care is necessary before analysing tissues from families, e.g., the Leguminosae, liable to contain such linkages.

Introduction

In forestry,[1] as in agriculture[2] and horticulture,[3] nitrogen constitutes an important nutrient element powerfully influencing productivity. In all three disciplines, the characteristics of the nitrogen economy embracing both plant and soil are of vital concern. But whereas in agriculture and horticulture attention frequently centres on growth responses to applied or fertiliser nitrogen, the tissue (usually the foliage) best reflecting nutritional status, in forestry (except for such special cases as nursery culture[4]) the absence of similar management makes the magnitude of nitrogen losses associated with different forms of logging an aspect of particular interest.[5, 6]

The estimation of such losses calls for the detailed sampling and analysis of a bulky and complex organism comprising much woody tissue. Some 94% of the dry weight of the aerial parts of an adult red pine (*Pinus resinosa* Ait.) tree, for example, are contained in the woody as distinct from foliar components, and of the total nitrogen present 45% or 148 kg./ha. are removed in the commercially utilisable timber.[7]

In the analytical determination of total nitrogen, the arborescent foliage presents no exceptional difficulty. Woody tissue, however, has three properties which collectively make it a most unusual and intractable material. It is very low in nitrogen, values of $0{\cdot}2\%$ for bark, $0{\cdot}3\%$ for twigs and $0{\cdot}05\%$ for wood being commonplace. It is bulky, especially when milled, and highly carbonaceous. Chemically it is far from homogeneous.

For routine work involving multiple determinations, therefore, the size and preparation of sample required render unsuitable all procedures, manual[8] and automatic,[9] based on the Dumas–Pregl principle and exclude all Kjeldahl digestions on a micro- or ultra-micro scale.[10] For Kjeldahl procedures on the feasible and convenient milligramme or semi-micro scale two main problems arise. The first[10–12] is the traditional one of selecting for the complex nitrogen compounds present catalysts and digestion conditions permitting a rapid and complete conversion to ammonium sulphate. And the second lies in critically adjusting the heating schedule and volume of sulphuric acid. Excessive initial heat and insufficient acid cause charring, spattering and localised overheating leading to erratic results, whereas too little heat retards digestion and too much acid prolongs the final distillation of ammonia by inconveniently increasing the amount of soda, hence overall volume of liquid, contained in the vacuum-jacketed chamber of a Parnas–Wagner apparatus.[13]

The purpose of this paper is threefold. First, to give the details of a milligramme-scale Kjeldahl procedure applicable on a trouble-free routine basis to all woody and foliar organs such as dead branches, twigs, bark, wood, cones, needles and leaves. Secondly, to present and discuss the results of recovery tests utilising this procedure when applied to a selection of amino-acids and other nitrogen-containing reference compounds likely to be present bound or free in arborescent tissues. And thirdly, to mention briefly experimental reasons for rejecting other procedures and techniques.

Experimental

I *The method*

Reagents

Wherever possible, chemicals of analytical or micro-analytical grade purity are used.

(1) Concentrated sulphuric acid, approximately 36 N.
(2) Potassium sulphate.
(3) Mercuric oxide.
(4) Base solution, 60% (w/v) in respect of sodium hydroxide (pellet form) and 5% (w/v) in respect of sodium thiosulphate.
(5) Boric acid, 1% (w/v).
(6) Standard hydrochloric acid, 0·01N.
(7) Standard nitrogen solution, containing 71·44 μg. of N/ml.: made by dissolving 1 g. of ferrous ammonium sulphate in 1 l. of 0·036N-sulphuric acid.
(8) Anhydrous sodium carbonate, oven-dried overnight at 150° before use.
(9) Mixed methyl red–methylene blue indicator; 0·2% (w/v) ethanolic methyl red and 0·2% (w/v) ethanolic methylene blue in the proportions 2:1 (v/v). Stable up to 1 month.
(10) Phenolphthalein indicator, 0·1% (w/v) in 70% (v/v) ethanol.
(11) Bromocresol green indicator, 0·1% (w/v) in 70% (v/v) ethanol.

Apparatus

(1) A Wiley No. 3 or other mill able to comminute woody tissues to particles not exceeding 1 mm. in size.
(2) Digestion rack with exhaust manifold able to accommodate 50-ml. borosilicate–glass Kjeldahl flasks. Coal-gas or preferably electrically (Electrothermal Co.) heated racks with individual temperature control for each flask are essential: racks fuelled by bottle-gas are unsuitable.
(3) Distillation unit of Parnas–Wagner design,[13] the usual silver condenser tube being replaced by one of silica (Vitreosil).
(4) A 10-ml. burette reading to 0·01 ml.

Procedure

A 100–300 mg. representative sample of woody tissue, previously oven-dried at 80° and milled to pass a 1 mm. sieve, is weighed into a 50-ml. Kjeldahl flask followed by 40 mg. of mercuric oxide and 4 g. of potassium sulphate. Five ml. of conc. sulphuric acid are added, the whole gently swirled and heating commenced. A suitable heating schedule for the electric racks is around 1·5 h. at thermostat setting No. 4, 0·5 h. at No. 6 and 2·0 h. at No. 10. Some variation in behaviour and rate of clearing occurs among the different woody tissues, but an essential general precaution is never to raise the temperature from the initial setting of No. 4 until the dark brown contents of the digestion flask have left the semi-solid for the viscous stage. Occasional rotation of the flasks may slightly accelerate clearing during the second stage of the digestion, but initially heating must never be so high that appreciable spattering of larger particles occurs on the upper parts of the bulb of the flask. Clearing to a colourless liquid usually takes place at settings 4 or 6. It is important to effect the quantitative transference of the clear digest to a 25-ml. volumetric flask before solidification sets in at room temperature when dissolution becomes tedious.

For the steam distillation of ammonia a suitable volume of the 25-ml. digest is transferred to the Parnas–Wagner apparatus followed by sufficient base solution to neutralise the acid and provide a moderate excess. The ammonia generated is trapped in 10 ml. of boric acid containing two drops of the mixed indicator. Distillation is complete after collection of 25 ml. of condensate. In the subsequent titration to a purple-grey end-point, 1 ml. of 0·01N-HCl = 140·1 μg of N.

Notes

(1) Although the volume of sulphuric acid destroyed by reaction with the carbonaceous material will vary from around 0·4 to 1·2 ml.,[10] the critical ratio of potassium sulphate to sulphuric

acid will always remain very close to 1·0 (w/v), the optimum for speedy digestion, and appreciably removed from values of 1·5 (w/v) and greater when loss of ammonia by volatilisation can occur.[11]

(2) Pre-treatment trials employing salicylic acid and sodium thiosulphate as transnitrating and reducing agents of possible oxidised forms of nitrogen[12] failed to yield statistically different results, when applied to foliage and bark. In any case the excessive frothing these reagents cause in the subsequent digestion would discourage their use.

(3) Yuen & Pollard's comprehensive study[14] has shown that 10 ml. of 1% boric acid are able to trap 1,000 μg. of N as ammonia with negligible loss. Distillation of larger amounts of ammonia, corresponding to 0·01N-HCl titres exceeding 7 ml., could require additional boric acid.

(4) The unavoidable replacement of the normal silver condenser tube by one of silica in the Parnas–Wagner apparatus gives a less steady distillation necessitating a more even supply of steam to avoid ' suck-back ' of condensate.

(5) Anhydrous sodium carbonate may be used to standardise the 0·01N-HCl, using 10-ml. aliquots of a solution containing 0·265 g. of the salt in 1 l. Titration with the acid is effected until the pink phenolphthalein colour is just dispersed, then after addition of two drops of bromocresol green indicator until the colour changes from blue to green. Finally carbon dioxide is expelled by boiling for 2 min. and the titration of the cooled solution taken to a permanent green. The 10-ml. aliquot of the sodium carbonate solution is exactly equivalent to 5 ml. of 0·01N-HCl.

(6) Both distillation unit and procedure may be conveniently tested with a 10-ml. aliquot of the standard ferrous ammonium sulphate solution. This generates ammonia equivalent to 5·10 ml. of 0·01N-HCl assuming a 100% purity of the salt.

(7) In routine work, digestions are conducted in duplicate with two blank digestions on the reagents alone for every ten unknowns. Duplicate distillations from the same 25–ml. digestion extract may be performed, 5–10-ml. aliquots requiring 0·01N-HCl titres of 0·5–5 ml. Duplicate distillations almost always agree and duplicate digestions occasionally differ only in the case of samples made up of anatomically different tissues—for example, twigs composed of wood and bark.

II *Recovery tests*

Reagents

All reagents listed previously were used in addition to the pure amino-acids and other nitrogen-containing reference standards shown in Table I. Each standard was the purest available commercially; source, grade and lot number being indicated.

Procedure

The procedure used was similar to that described above except for the following modifications. From 20 to 70 mg. of standard substance were taken with 10–20 mg. of mercuric oxide, 2 g. of potassium sulphate and 2 ml. of conc. sulphuric acid. Except for nicotinic acid and canavanine which do not char, clearing time was somewhat shorter, the entire heating schedule being 60–100 min. at thermostat setting No. 4, 20–30 min. at setting No. 6, and the same final 2·0 h. at setting No. 10. For convenience digests were made up to 50 ml. and aliquots of 4 ml. distilled: titres of 0·01N-HCl required were around 5 ml.

Results

The results of the recovery tests are shown in Table I.

With two exceptions, all results are very satisfactory, no percentage recovery being in error by more than ±0·6%, and they are precise in that all coefficients of variation are less than 0·6%. Clearly, some departure from 100% recovery may be expected, for the standard substances themselves are neither exactly 100% pure nor can errors be altogether excluded from the various experimental operations such as the acid standardisation. Nevertheless, there is no suggestion that the digestion conditions fail to give other than an almost complete conversion to ammonium sulphate. And corroboration of this experience stems from the studies of Bremner,[11] Navellier & Alegre,[15] and Sietz,[16] all of whom tested the efficiency of mercury catalysts upon various

Table I

Recovery of nitrogen from various standard reference compounds

Compound	Nitrogen content, % Theoretical	Found*	Coefficient of Variation, %	Recovery, %
L-Arginine hydrochloride, (Calbiochem 101347) (aliphatic amino acid with guanidino-N)	26·60	26·61 ± 0·083	0·31	100·04
L-Histidine hydrochloride, (Calbiochem 103821) (aliphatic amino acid, with imidazole-N)	20·04	20·16 ± 0·032	0·16	100·60
Hydroxy-L-proline, (Calbiochem 104706) (heterocyclic amino-acid with ring-N)	10·68	10·72 ± 0·055	0·51	100·37
L-Tryptophan, (Calbiochem 46227) (heterocyclic amino acid with indole-N)	13·72	13·69 ± 0·045	0·33	99·80
Adenosine, (NBC 3044) (heterocyclic amino-acid with purine-N)	26·21	26·34 ± 0·093	0·35	100·50
Allantoin, (BDH 24909) (heterocyclic amino-acid with ring and ureido-N)	35·43	35·56 ± 0·120	0·33	100·37
Urea, (A.R., BDH 761195)	46·65	46·88 ± 0·190	0·40	100·49
Phenylthiourea, (M.A.R., BDH 744480)	18·41	18·47 ± 0·062	0·34	100·36
DL-Methionine, (NBC 6363) (sulphur amino-acid)	9·39	9·39 ± 0·037	0·39	100·07
L-Cystine, (NBC 5338) (sulphur amino-acid)	11·66	11·67 ± 0·054	0·46	100·08
Nicotinic acid, (U.S.P., Calbiochem 45243) (pyridine-N)	11·38	11·35 ± 0·034	0·30	99·76
8-Hydroxyquinoline (M.A.R., BDH 2344280) (quinoline-N)	9·65	9·67 ± 0·023	0·24	100·20
L-Canavanine, (Calbiochem 30351) (aliphatic amino acid, guanidoxy-N)	20·43	17·68 ± 0·290	1·64	85·34
Diphenylthiocarbazone, A.R., BDH 705392) (azo-N)	21·86	14·12 ± 0·091	0·64	64·62

*Mean of ten digestions ± standard deviation

B.D.H. = British Drug Houses, Toronto; Calbiochem = California Corp. Biol. Res., Los Angeles;
N.B.C. = Nutritional Biochemical Corp., Cleveland

nitrogen standards. However, the range of compounds or nitrogen-containing structures tested here typifies those found in predominant amounts in higher plants and embraces several well known for their resistance to breakdown, e.g. tryptophan and nicotinic acid.

The failure of the method in the case of canavanine (N–O linkage) and diphenylthiocarbazone (N–N bond) is not unexpected. Both are included among that particular group of compounds— nitro, nitroso, hydrazo, azo, azoxy, oximes and hydrazines—which are not susceptible to a simple unmodified Kjeldahl procedure,[12, 17] and such compounds pose special problems, for no one modification may prove satisfactory for all. The salicylic–thiosulphate pretreatment described above, for example, improved the nitrogen recovery to 90·0% in the case of canavanine, but only to 68·3% for diphenylthiocarbazone. Fortunately, nitrogenous substances containing such linkages are seldom found in higher plants,[18, 19] but there are exceptions which are discussed later.

III *Rejected procedures*

At the outset of this investigation procedures excluding mercury from the digestion mixture were considered because of the silver condenser tube of the Parnas–Wagner apparatus. The highly carbonaceous nature of woody tissues gave oxidative attacks based on potassium permanganate[20] or hydrogen peroxide[21] especial appeal but in spite of their speed they were thought to demand excessive attention and skill to be suitable for trouble-free routine operation. Moreover, although both oxidants give results similar to those with selenium and mercury catalysts for coffee leaves[22] and grasses[23] respectively, erratic and low results have been reported using potassium permanganate with soils[11] and slightly low results for hydrogen peroxide with various agricultural materials[24] and standard amino-acids.[25, 26]

Semi-micro adaptations of three apparently promising procedures were selected for critical comparison. The first utilised a potassium sulphate–copper sulphate–selenium catalyst in the proportion successfully used for soils by Bremner,[11] except that the ratio of catalyst to sulphuric acid was raised to 4 g./5 ml. The second was based on the potassium sulphate–vanadium pentoxide–selenium catalyst recommended for coals by Edwards,[27] the ratio of mixed catalyst to acid being as above. And the third more uncommon method was the one favoured by Ward & Johnston[28] for plant materials, in which 5 ml. of sulphuric acid–phosphoric acid–copper sulphate–selenous acid mixture are used, prepared in the proportions 75 ml./25 ml./1 g./1·2 g. In all cases 300 mg. of woody tissue or 20–70 mg. of standard nitrogen compound were weighed into 50-ml. flasks and digestions continued for 2 h. after clearing, as with the mercuric oxide method.

Aside from accuracy, all three rejected procedures exhibited operational shortcomings. In two, a homogeneous catalyst mixture necessitated pulverisation in a mortar and, in the third, dissolution of the copper sulphate and selenous acid in phosphoric acid was protracted, features which expose reagents to possible contamination. Digestions were smooth in the Bremner and Edwards procedures but in the former, undesirable build-up of selenium occurred on the necks of the digestion flasks and exhaust manifold. Both with and without glass beads the Ward & Johnston digestions were dangerously eruptive, whilst attack on the flasks by the phosphoric acid and the residual incrustation of salts created further problems. There was very little difference in speed among all four methods and all blanks were negligible.

Results

The recovery of nitrogen from phenylthiourea and the nitrogen contents of various representative woody tissues obtained by the three trial procedures and the proposed mercuric oxide method are shown in Table II.

Edwards' procedure was immediately rejected because results were both low and erratic; a surprising outcome in view of Edwards' almost identical results with coals irrespective of whether a vanadium pentoxide/selenium or mercuric sulphate/selenium catalyst were employed. Bremner's procedure gave excellent reproducibility but consistently low results, a conclusion not at variance with other workers' experience of selenium *vis-à-vis* mercury.[8, 10, 16] The results by Ward & Johnston's procedure came nearest to those obtained with mercuric oxide, but more than

Table II

*Nitrogen recovery and nitrogen content of woody tissues by various Kjeldahl procedures**

Method**	Nitrogen standard, phenylthiourea, recovery, %	Wood tissues, *** nitrogen content, %			
		Needles	Twigs	Bark	Wood
Proposed, K_2SO_4/HgO	100·36	1·047	0·407	0·196	0·152
Bremner,[11] $K_2SO_4/CuSO_45H_2O$/Se	97·05	0·955	0·387	0·178	0·139
Edwards,[27] K_2SO_4/V_2O_5/Se	71·68	0·327	0·097	0·036	0·019
Ward & Johnston,[28] $H_3PO_4/CuSO_4$, $5H_2O/H_2SeO_3$	99·58	1·039	0·410	0·189	0·149

*All figures are means of triplicate digestions and concordant distillations
**Modified as explained in the text
***Sources are detailed in Table V

triplicate digestions of woody materials would be necessary to give a reliable estimate of the apparently small percentage discrepancy. The closeness of the results, especially with phenylthiourea, and the recent claim[29] that the method gives good results with heterocyclic nitrogen compounds based on isatin, prompted further trials, the results of which are shown in Table III.

Obviously the Ward & Johnston digestion was less able to break down more resistant nitrogen compounds which, taken with its eruptive nature and other shortcomings, left little point to its further development.

Notwithstanding the greater accuracy of the proposed mercuric oxide procedure and its satisfactory use for many hundreds of woody tissue samples, difficulties have been reported[11] in the distillation of digest extracts based on mercury catalysts. A survey of 15 recent procedures incorporating mercury revealed a variable but generally high rate of usage ranging from 115 to 250 mg. of mercuric oxide per 5 ml. of concentrated sulphuric acid employed. Only in two cases[30, 31] was the weight less than that of the 40 mg. proposed—18 and 5 mg. respectively—but in both of these mercury was only part of the catalytic mixture. That a 40-mg. rate is adequate is suggested by the results in Table IV, but that there probably exists for more resistant compounds a lower inadequate rate may also be inferred.

It was concluded that most previous procedures based on a mercury catalyst employed excessive quantities, since an amount corresponding to 40 mg. of mercuric oxide per 5 ml. of sulphuric acid appeared sufficient for the most resistant nitrogen compounds.

Results

Precision

The reproducibility of the proposed method is shown in Table V together with estimates of the experimental replication required to give means of desired levels of precision.

Table III

*Nitrogen recovery from reference compounds by two Kjeldahl procedures**

Method	Nitrogen recovery, % Reference compound***			
	Nicotinic acid	L-Tryptophan	8-Hydroxyquinoline	L-Canavanine
Proposed, K_2SO_4/HgO	99·76	99·80	100·20	85·34
Ward & Johnston,** $H_3PO_4/CuSO_4$, $5H_2O/H_2SeO_3$	64·67	96·81	90·40	83·11

*All figures are means of at least three digestions
**Modified as explained in the text
***Further specified in Table I

Table IV

*Nitrogen recovery from resistant compounds with different rates of mercuric oxide catalyst**

Rate, mg. of HgO per 5 ml. of H_2SO_4	Nitrogen recovery, % Reference compounds**			
	Nicotinic acid	L-Tryptophan	8-Hydroxyquinoline	Phenylthiourea
25	97·56	97·42	99·40	99·70
40	99·82	100·06	99·96	100·34
100	99·76	99·80	100·20	100·36

*All figures are means of at least three digestions
**Further specified in Table I

Table V

Precision of the method for various types of woody tissue

Nature of sample*	Nitrogen content, %**	Coefficient of variation, %	Sample size required for an error***	
			$\gtrless \pm 5\%$	$\gtrless \pm 10\%$
Needles (representative of centre part of crown, tree No. 13)	1·047 ± 0·014	1·34	<1	<1
Twigs (wood plus bark but no needles, representative of centre and lower parts of crown, tree No. 24)	0·564 ± 0·008	1·38	<1	<1
Apex of mainstem up to 3 cm. in diam. (wood plus bark but no needles, tree No. 24)	0·407 ± 0·012	2·94	<3	<1
Bark (representative of lower bole 21·01–26·52 cm. in dia., tree No. 19)	0·196 ± 0·0018	0·92	<1	<1
Dead branches (cut from lower mainstem, tree No. 24)	0·196 ± 0·0059	3·01	<3	<1
Wood (from upper mainstem 3–7 cm. in dia., tree No. 17)	0·152 ± 0·0035	2·30	<2	<1
Wood (from lower mainstem 16·43–17·58 cm. in dia., tree No. 2)	0·063 ± 0·0022	3·49	<4	<1

*All from red pine (*Pinus resinosa* Ait.), aged 8 years (No. 17), 21 years (No. 13), 34 years (No. 2), 47 years (No. 24) and 81 years (No. 19).
**Mean of five digestions ± standard deviation
***Calculated from the formula $N = \frac{\sigma^2 \times t^2_{.05}}{(\bar{x} \times \pm \text{P.E.})^2}$ where N = sample size, σ = standard deviation, $t_{.05}$ = value for appropriate number of observations at a probability of 0·05, $\bar{x}$ = mean, and P.E. = the permissible error of a sample mean from the true mean[32]

Coefficients of variation are all less than 4% and single determinations would not deviate from a true mean by more than ±10%. A precision of ±5% appears obtainable for most tissues with duplicate or triplicate digestions.

Applications

Table VI indicates the variation in nitrogen content which is typically met among the component woody tissues of a tree and the variability from tree to tree within a plantation. Nitrogen contents decrease in the order needles > bark > wood and from older to younger tissues.

Percentage nitrogen contents may be combined with oven-dry weight data to provide the absolute contents of the right-hand column and these may be summated as in Table VII to show nitrogen removals from the forest associated with different forms of logging.

Discussion

Three main reasons suggest that the proposed semi-micro Kjeldahl procedure is satisfactory for the determination of nitrogen in woody tissues. First, it gives good reproducibility when

Table VI

Content and distribution of nitrogen within 34-year-old plantation-grown red pine trees

Component organ of tree	Nitrogen content, %*	Oven-dry weight of organ, kg.	Absolute nitrogen content of organ, g.*
Dead branches	0·205±0·021	5·52	11·36±2·85
Mainstem wood:**			
3–7 cm. dia. (overbark)	0·100±0·0104	1·47	1·47±0·27
7–9 cm. ,, ,,	0·089±0·006	2·00	1·78±0·28
9–13 cm. ,, ,,	0·079±0·0081	13·12	10·38±1·41
13–15·3 cm. ,, ,,	0·066±0·0056	12·00	7·91±1·29
15·3–16·9 cm. ,, ,,	0·064±0·0043	14·07	9·00±1·12
16·9–19·7 cm. ,, ,,	0·063±0·0041	15·38	9·69±1·39
19·7–21·8 cm. ,, ,,	0·064±0·0036	15·04	9·62±1·86
Mainstem bark:**			
3–7 cm. dia. (overbark)	0·500±0·034	0·34	1·71±0·45
7–9 cm. ,, ,,	0·396±0·030	0·37	1·46±0·38
9–13 cm. ,, ,,	0·330±0·045	1·84	6·09±0·78
13–15·3 cm. ,, ,,	0·281±0·028	1·43	4·01±0·64
15·3–16·9 cm. ,, ,,	0·254±0·026	1·61	4·08±0·78
16·9–19·7 cm. ,, ,,	0·232±0·012	1·86	4·32±0·77
19·7–21·8 cm. ,, ,,	0·227±0·020	1·92	4·36±1·29
Large live branches (wood plus bark, no needles)	0·187±0·015	4·60	8·58±1·83
Twigs and small branches (wood plus bark, no needles)	0·394±0·033	2·67	10·50±2·15
Needles	1·096±0·044	7·02	76·95±13·77
Total (all aerial organs)		102·26	183·27±23·86

*Mean of ten average trees on a 0·1-ha. plot ± standard deviation
**The various diameter classes represent approximately successive 2-m. lengths from apex to stump: the 0–3-cm. category is included in twigs.

Table VII

Removals of nitrogen from a forest site associated with different forms of logging (based on the data of Table VI)

Tree organs removed*	Removal of nitrogen g./tree	kg./ha.**
Peeled logs > 9 cm. dia.	46·60	89·6
Unpeeled logs > 9 cm. dia.	69·46	133·5
' Full-tree ' logging; all aerial organs	183·27	352·3

*Volumes are: wood, 327·1 m.³/ha.; bark 57·4 m.³/ha.
**Based on 1922 trees/ha.

applied to natural materials which by all agricultural comparisons[33] are extremely low in nitrogen. Secondly, it gives higher values for such materials compared with those obtained by three other recently described procedures. Thirdly, it gives good recoveries from a variety of nitrogen reference compounds whose structures or composition are likely to be predominantly present in plant tissues.[18, 34]

The limitation of the procedure is no greater—perhaps somewhat less—than that inherent in all Kjeldahl procedures, viz., their inability, without special modification, to recover all nitrogen bound in N–N or N–O linkages. Whilst the appraisal of pre-treatment modifications is outside the scope of this study, it may be salutary to indicate occurrences of such linkages where nitrogen content could be underestimated.

The first case is nitrate-N. Normally this is present in negligible amounts,[34] but where it occurs through heavy nitrate-fertiliser application[23] incomplete recovery may be expected. The second but so far rare occurrence is that of nitro-compounds; β-nitropropionic acid, as a glycoside, in the wood and bark of *Hiptage mandoblata* and the berries of the karaka tree (*Corynocarpus laevigata*),[35] the aristolachia acids in the climber *Aristolachia clematis*,[36] and the cinnamon-like phenyl-1-nitroethane from the wood and bark of the Brazilian Lauraceae *Ocotea pretiosa* and *Aniba canelilla*.[37] The third occurrence, rapidly increasing in frequency, is that of the alkaloid N-oxides.[38] Whereas the alkaloids themselves may be no more resistant than tryptophan and

nicotinic acid,[16] the amine oxide co-ordinate bond may exhibit a difficulty comparable with that for similar linkage between one of the oxygen atoms and nitrogen in the nitro-group. Generalisation is difficult because of the variability in alkaloid structures and the greatly varying stability demonstrated for the amine oxide group[39] but caution would be necessary with herbaceous members of the Boraginaceae and Compositae and arborescent members of the Leguminosae which families exhibit a high ratio of alkaloid N-oxide to alkaloid.[40–42] A fourth instance, the azoxy-aglycone moiety of macrozamin, appears confined to certain Australian Cycadaceae,[43] whilst the last group, guanidoxy, is represented naturally solely by canavanine but seems widely distributed within the Leguminosae,[44] mainly in the seeds[45] but sometimes in the leaves.[46]

Collectively these occurrences do not warrant a general pretreatment modification to the Kjeldahl procedure or its abandonment in favour of the Dumas method. Not only is there probably no simple pre-treatment satisfactory for all difficult cases,[12] but the Dumas approach has problems of its own, not the least of which is a necessity to read accurately a volume of nitrogen varying from 0·10 to 1·50 ml. generated from a sample weighing 0·15 g., the maximum for a combustion boat of 0·8-ml. volume. The results do suggest caution, however, in the application of the method to certain plant tissues, particularly those from the Leguminosae, and this is of especial importance in view of the major role of the Leguminosae in considerations of nitrogen–balance and economy,[46] both in natural and man-managed plant communities.

It may be noted that knowledge of the nitrogen content of arborescent species has remained peculiarly limited[5, 6] partly through an early necessity to apply the laborious pre-Kjeldahl procedures of Dumas or Varrentrapp & Will[47] and partly later through the difficulty of applying Kjeldahl procedures to woody tissues. It is hoped the proposed method will facilitate the latter.

Acknowledgments

Mr. G. V. Lafrance performed all experiments concerned with plant materials and Mr. I. K. Morrison most of the recovery tests with reference standards.

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Received 22 February, 1965; amended manuscript 14 March, 1965

References

[1] Tamm, C. O., & Carbonnier, C., *K. Skogs. Lantbr Akad. Tidskr.*, 1961, **100**, 95
[2] Cooke, G. W., *J. Sci. Fd Agric.*, 1958, **9**, 761
[3] Reuther, W., Embleton, T. W., & Jones, W. W., *Annu. Rev. Pl. Physiol.*, 1958, **9**, 175
[4] Benzian, B., *J. Sci. Fd Agric.*, 1959, **10**, 637
[5] Rennie, P. J., *Plant & Soil*, 1955, **7**, 49
[6] Leyton, L., in ' Encyclopedia of Plant Physiology ', 1958, Vol. IV, p. 1026 (Ed. Ruhland, W.) (Berlin: Springer-Verlag)
[7] Rennie, P. J., unpublished data
[8] Kirsten, W., in ' Comprehensive Analytical Chemistry ', 1960, Volume IB, p. 467 (Eds. Wilson, C. L., & Wilson, D. W.) (London: Elsevier)
[9] Gustin, G. M., *Microchem. J.*, 1960, **4**, 43
[10] Kirk, P. L., *Analyt. Chem.*, 1950, **22**, 354
[11] Bremner, J. M., *J. agric. Sci. Camb.*, 1960, **55**, 11
[12] Taras, M. J., in ' Colorimetric Determination of Nonmetals ', 1958, p. 75 (Ed. Boltz, D. F.) (London: Interscience)
[13] Jones, A. S., as reference 8, Vol. IB, p. 495
[14] Yuen, S. H., & Pollard, A. G., *J. Sci. Fd Agric.*, 1953, **4**, 490
[15] Navellier, P., & Alegre, J., *Ann. Falsif., Paris*, 1960, **53**, 542
[16] Sietz, F. G., *Chemikerztg*, 1960, **84**, 362
[17] Albert, J., in ' Proc. of 1961 International Symposium on Microchemical Techniques ', 1962, Vol. 2, p. 527 (Ed. Cheronis, N. D.) (London: Interscience)
[18] McKee, H. S., ' Nitrogen Metabolism in Plants ', 1962 (Oxford: Clarendon Press)
[19] Browning, B. L., ' The Chemistry of Wood ', 1963 (New York: Wiley)
[20] Beet, A. E., *J. appl. Chem.*, 1954, **4**, 373
[21] Pinion, L. C., & Farmer, R. H., *Chem. & Ind.*, 1958, p. 919
[22] Robinson, J. B. D., *Analyst*, 1956, **81**, 316
[23] Ekpete, D. M., & Cornfield, A. H., *Analyst*, 1964, **89**, 670
[24] Hübsch, H., & Nehring, K., *Z. anal. Chem.*, 1960, **173**, 278
[25] Ann. Rep. Forest Products Research Laboratory for 1962, 1963, p. 10 (London: H.M.S.O.)
[26] McKenzie, H. A., & Wallace, H. S., *Aust. J. Chem.*, 1954, **7**, 55
[27] Edwards, A. H., *J. appl. Chem.*, 1954, **4**, 330
[28] Ward, G. M., & Johnston, F. B., ' Chemical Methods of Plant Analysis ', 1962, Publ. 1064, Canada Dept. Agric. (Ottawa: Queen's Printer)
[29] Čepčiansky, I., & Chromcovà, L., *Chem. Prům.*, 1959, **9**, 188
[30] Jackson, M. L., ' Soil Chemical Analysis ', 1958 (New Jersey: Prentice-Hall)

References (*cont.*)

[31] Jacobs, S., *Nature, Lond.*, 1959, **183**, 262
[32] Snedecor, G. W., ' Statistical Methods ', 1957, 5th Edn. (Ames: Iowa State College Press)
[33] McCance, R. A., & Widdowson, E. M., ' The Composition of Foods ', 1960 (Med. Res. Counc. Spec. Rep. Series No. 297, 3rd Edn.) (London: H.M.S.O.)
[34] Steward, F. C., & Durzan, D. J., in ' Plant Physiology: An Advanced Treatise ', 1965, Volume IVA, (Ed. Steward, F. C.) (New York: Academic Press)
[35] Carter, C. L., & McChesney, W. J., *Nature, Lond.*, 1949, **164**, 575
[36] Pailer, M., & Schleppnik, A., *Mh. Chem.*, 1957, **88**, 367
[37] Gottlieb, O. R., & Magalhaes, M. T., *J. org. Chem.*, 1959, **24**, 2070
[38] Willaman, J. J., & Schubert, B. G., ' Alkaloid-Bearing Plants and their Contained Alkaloids ', 1961, Tech. Bull. No. 1234 (Washington: U.S. Dept. of Agriculture)
[39] Taylor, E. C., & Loeffler, P. K., *J. org. Chem.*, 1959, **24**, 2035
[40] Fish, M. S., Johnson, A. M., & Horning, E. C., *J. Amer. chem. Soc.*, 1955, **77**, 5892
[41] Crow, W. D., & Michael, M., *Aust. J. Chem.*, 1957, **10**, 177
[42] Bohlmann, F., Rahtz, D., & Arndt, C., *Chem. Ber.*, **91**, 2189
[43] Riggs, N. V., *Aust. J. Chem.*, 1954, **7**, 123
[44] Bell, E. A., *Biochem. J.*, 1958, **70**, 617
[45] Birdsong, B. A., Alston, R., & Turner, B. L., *Canad. J. Bot.*, **38**, 499
[46] Anon., *Chem. & Ind.*, 1959, p. 171
[47] Varrentrapp, F., & Will, H., *Liebigs Ann.*, 1841, **39**, 257

THE SURFACE LIPIDS OF FRESH AND PROCESSED RAISINS

By F. RADLER

The surface lipids of the grapes from varieties used for the production of Sultanas, raisins and currants were extracted with chloroform. A further separation of the extracts with light petroleum yielded the insoluble ' hard wax ' which consists mainly of oleanolic acid, and the soluble ' soft wax '. This fraction was separated by chromatography on aluminium oxide into the hydrocarbon, ' ester–aldehyde ', alcohol and acid fractions. The composition of the hydrocarbon and alcohol fractions of the surface wax of fresh grapes and the composition of the saponifiable fraction of lipids from dried and processed grapes has been determined by gas/liquid chromatography. The addition of oils to dried grapes can be detected and determined by chromatography on aluminium oxide or saponification and estimation of the fatty acids.

Introduction

Dried and processed raisins generally do not contain their natural lipids only but certain amounts of lipid processing aids are present. The amounts and composition of these additives present on processed raisins depends on the grape variety and the drying and processing methods. The methods used for the production of raisins throughout the world have been summarised by Winkler[1] and Cruess,[2] and the Australian drying methods have been recently reviewed by Permezel.[3]

Two different types of lipid processing aids are added to the fruit. To increase the drying rate and improve the colour of the final product, Sultanas are dipped into a cold emulsion of ' dipping oil ' in potassium carbonate.[4] This is the general practice in Australia. During this treatment the overall lipid content of the fruit is increased,[5, 6] as part of the oil is absorbed by the grape surface. Grape varieties, other than Sultanas, are usually dried without oil treatment (e.g. currants are dried without any treatment and Gordo and Waltham Cross are dipped in hot caustic soda).

After the cleaning, capstemming and washing procedures, oil is added to the dried fruit in order to improve the appearance and to avoid stickiness during storage and transport. This final dressing is particularly important for raisins which have been mechanically deseeded. Mineral oil is the most widely used dressing oil for dried grapes in Australia, but suitable alternative oils of plant or animal origin have been tested.[7]

Up to the present time very little information is available about the amounts of lipid additives remaining on processed dried grapes. Mineral oil can be detected and determined by column chromatography[8, 9] or by thin-layer chromatography.[10, 11] The determination of other additives is complicated by the fact that they are similar or identical with the natural lipids of the grape surface. The composition of the cuticular waxes of Sultanas has been analysed by Radler & Horn,[12] and from the results the composition of the surface lipids of fresh currants, raisins and Sultanas was determined. The analysis of processed dried grapes yielded information about changes of the surface lipids during processing and the amounts of processing aids remaining on the fruit.

Experimental

Fresh and dried grapes of the varieties Sultana (Thompson's Seedless), Waltham Cross (Rosaki), Muscat Gordo Bianco and Currant were obtained from the experimental vineyards of the Horticultural Research Section, Merbein, Victoria, in the drying season 1964. Processed fruit was kindly supplied by local packing sheds. Commercial ' dipping oils ' were used.

The surface wax of fresh grapes was extracted by immersion in cold chloroform (3 × 10 sec.) as suggested by Martin.[13] Dried grapes were extracted for 3 h. with chloroform in a Soxhlet apparatus.

The content of oleanolic acid in the ' whole wax ' was determined by the Liebermann–Burchardt reaction according to Brieskorn & Hofmann.[14] The ' whole wax ', also containing lipid processing aids, was separated with light petroleum into insoluble ' hard wax ' and soluble ' soft wax '. The ' hard wax ' consists mainly of oleanolic acid, the soluble fraction contains the fruit wax components and lipid processing aids. The separation and determination of the wax components by column chromatography on aluminium oxide, thin-layer and gas–liquid chromatography were performed as described in detail by Radler & Horn.[12] All fractions obtained were checked by thin-layer chromatography. Saponification of the lipid extracts was effected according to Matic & Horn;[15] separation of unsaturated fatty acids and esters of drying oils as mercury adducts (see Mangold[16]); and determination was made of free and bound sulphur.[17] The dipping oils, consisting mainly of ethyl esters and fatty acids, were methylated with diazomethane. The ester mixture thus obtained was separated by gas–liquid chromatography and the content of free acids in the oils determined from the amount of methyl esters present.

Results

The overall composition of the surface wax obtained by chloroform extraction from fresh grapes of the important drying varieties is compiled in Table I. The main constituent of the surface wax of these grapes is oleanolic acid, which amounts to three-quarters and more of the surface wax of Sultanas, Waltham Cross and Gordos, and about half of the wax of Currants. The light petroleum–soluble fraction of the wax was separated by chromatography on aluminium oxide into hydrocarbons, an ' ester–aldehyde ' fraction, alcohols and fatty acids. The aldehydes present in the cuticle of fresh grapes are mostly converted to compounds of high molecular weight

Table I

The main components of the surface lipids of fresh grapes from varieties used for drying
(In brackets: % of light petroleum-soluble fraction of wax)

Fraction	Grape Variety			
	Sultana	Waltham Cross	Gordo	Currant
Oleanolic acid, %	75	84	75	44
Light petroleum soluble, %	24	14	23	65
Hydrocarbons, %	<1 (2)	<1 (3)	<1 (1)	<2 (3)
Esters and aldehydes, %	5 (0)	3 (24)	5 (22)	7 (12)
Alcohols, %	10 (43)	7 (52)	9 (40)	29 (52)
Free fatty acids, %	2 (8)	1 (6)	2 (9)	6 (11)
Not eluted, %	6 (27)	2 (15)	6 (28)	12 (22)

by chromatography on alumina[12] and are then eluted together with the esters and partly retained on the column together with some non-lipid material and some of the fatty and oleanolic acids. The surface waxes of the varieties used for the production of dried grapes appear to be similar.

The compositions of the hydrocarbon and alcohol fractions as obtained by gas–liquid chromatography are shown in Table II. The hydrocarbons range from about n-C_{20} to n-C_{35}, the uneven numbered members being the most abundant. Slight differences can be observed between the varieties; Sultana and Currant varieties containing more of the n-C_{25} and n-C_{27}, Waltham Cross and Gordo containing more of n-C_{27}, n-C_{29} and n-C_{31} homologues. The alcohols range from about n-C_{20} to n-C_{34}. The main component is n-C_{26} with the exception of Waltham Cross, which contains almost equal quantities of n-C_{26} and n-C_{28}. The alcohol fraction contains varying amounts of an unidentified substance (possibly triterpene alcohol or sterol) which is not volatile and not likely to interfere with the separation by gas liquid chromatography.

For the production of dried grapes, Currants are dried without treatment, Sultanas are either sun-dried (in California) or cold-dipped (in Australia). Gordos and Waltham Cross are usually dipped in hot caustic soda (2–4 sec. at 220–190°F). Quantitative figures for the grape surface lipids are presented in Table III. Sultana grapes dried after cold-dipping contain about 1 g. more lipid material per kg. than sun-dried fruit. This increase of lipid material is mainly found in the saponifiable part of the light petroleum–soluble fraction. The washing of the grapes in the packing house seems to remove some of this added lipid. Treatment of the fruit with mineral oil leads to a marked increase in total extractable lipids, and the mineral oil is found in the unsaponifiable part. Waltham Cross and Gordo grapes, which are dipped in hot caustic soda, lose some of their lipids; the highest loss seems to occur in the saponifiable fraction of the light petroleum–soluble lipids where 19 and 28% are removed by dipping.

Saponification of the petroleum–soluble material of untreated grapes yields about half unsaponifiable and saponifiable material. The total amount is usually not recovered after saponification, indicating the presence of some non-lipid material. The aldehydes are destroyed by saponification[18] and are mainly found as polymerisation products in the unsaponifiable part. Oleanolic acid is present in the light petroleum–soluble fraction in varying amounts, and therefore high and varying amounts of saponifiable material are found.

The oils used for the dipping of Sultana grapes are commercial products, consisting mainly of fatty acid esters. Martin & Stott[5] published the general composition of two such dipping oils. A more detailed analysis of two dipping oils as used today is presented in Tables IV and V.

Table II

Composition of the hydrocarbon and free alcohol fractions of the surface lipids of fresh grapes of varieties mainly used for drying

(In % of the total fractions; o = <0·3%; + = <1%)

Carbon No.	Hydrocarbons, %				Alcohols, %			
	Sultana	Waltham Cross	Gordo	Currant	Sultana	Waltham Cross	Gordo	Currant
<20	+	o	+	+	o	+	+	+
20	+	+	o	+	+	o	o	+
21	2	+	+	2	+	o	o	+
22	3	1	+	1	1	1	2	3
23	7	4	3	13	+	+	+	1
24	7	4	3	3	19	8	14	24
25	19	11	17	28	2	1	2	2
26	5	4	4	3	39	21	34	34
27	18	18	22	36	1	2	3	2
28	4	4	4	2	17	24	24	22
29	15	21	19	9	1	3	1	+
30	3	4	3		7	9	7	6
31	11	24	20	1				
32	1	2	1		5	12	6	3
33	+	2	2	o				
34					o		o	
35	+		+					
Sum of minor unknown peaks	2	+	1	1	7	18	6	2

Table III

The lipid extracts of dried and processed Sultanas and raisins

(g./kg. of fruit)

	Total chloroform extract	Light petroleum-insoluble of chloroform extract (oleanolic acid)	Light petroleum-soluble of extract	Unsaponifiable of petroleum-soluble fraction	Saponifiable of light petroleum-soluble fraction
Sultanas					
Untreated (sun dried)	1·36±0·06*	0·84±0·13	0·51±0·10	0·30	0·20
Dipped	2·27±0·13	0·92±0·09	1·36±0·15	0·44	0·93
Dipped and washed	1·92	0·86	1·06	0·42	0·61
Dipped and oiled	4·50	1·12	3·38	2·98	0·39
Walthams					
Untreated	1·31	0·68	0·63	0·26	0·32
Hot dipped	1·06	0·57	0·49	0·22	0·26
Gordos					
Untreated	1·73	0·89	0·84	0·31	0·46
Hot Dipped	1·49	0·80	0·69	0·26	0·33

*standard deviation

Besides some volatile matter and ash, such dipping oils consist almost entirely of fatty acids and their esters, as about 80% of the oils are saponifiable (Table IV). The iodine numbers indicate that less than half of the acids are unsaturated. The chain-length distribution is shown in Table V. Main constituents of two oils are the n-C_{18} acids which are mainly present as ethyl esters (Table V).

Table IV

The composition of two commercial 'dipping oils'

	Oil A	Oil B
Volatile matter, %	10·6	10·5
Ash, %	1·85	3·94
Total sulphur, mg. S/g.	4·9	10·7
Free sulphate, mg. S/g.	0·85	2·74
Unsaponifiable, %	1·23	0·38
Saponifiable, %	78·5	77·2
% of fatty acids not esterified	15	10
Iodine number	39	40

Table V

The composition of free fatty acids and ethyl esters of fatty acids (saturated and unsaturated) in two commercial 'dipping oils'

(% of total fatty acids and esters; o = <0·3%; + = <1%)

Carbon no. of fatty acids*	Oil A		Oil B	
	fatty acid ethyl ester	free acid	fatty acid ethyl ester	free acid
<12	o		o	
12	+		+	
14	4	+	4	+
15	+		+	
16	23	1	21	2
17	+		1	
18	50	8	54	12
20	9		+	
20			+	
Unknown	4		4	

* The acids were separated by gas–liquid chromatography on a Chromosorb column coated with silicone grease, which does not separate saturated from unsaturated acids. Methylation of the oils and separation of the mercuric adducts by thin-layer chromatography revealed that most of the free acids of the oils are unsaturated.

The composition of the fatty acids in the saponifiable part of the petrol-soluble fraction of the grape surface lipids is shown in Table VI. The acids range from about n-C_{14} to n-C_{32}. As

Table VI

Composition of the acid fraction (saponifiable) of the lipid extracts from dried and processed grapes
(expressed in % of the n-acids; o = <0·3%; + = <1%)

Carbon No.	Sultanas			Waltham Cross		Gordo	
	Sun dried	Dipped	Dipped and washed	Sun dried	Hot dipped	Sun dried	Hot dipped
<14	3	o	o	o			
14	2	2	2	+	o	+	1
15	+	+	+	o	o	o	o
16	4	18	16	12	6	11	10
17	+	1	+	+	+	+	+
18	6	37	29	26	14	28	23
19	1	+	+	+	+	+	+
20	12	4	4	12	17	9	9
21	1	+	+	+	+	+	+
22	11	5	4	8	9	6	6
23	2	1	1	+	+	+	+
24	11	5	5	7	8	8	9
25	3	+	1	+	+	1	+
26	12	5	6	6	9	9	11
27	2	+	1	+	1	+	+
28	14	7	11	11	16	10	12
29	1	+	1	+	1	+	+
30	8	5	8	7	9	7	7
31	1	+	+	+	+	+	+
32	5	4	7	4	7	6	6
Sum of minor unknown peaks	1	3	2	4	+	2	2

expected, the content of n-C_{16} and n-C_{18} acids is highly increased by dipping. The washing of Sultana grapes appears to remove some of these acids, but the effect is not very appreciable. Hot dipping of Waltham Cross and Gordo grapes seems to lower the content of n-C_{16} and n-C_{18} acids. The content of these acids is much higher in fresh Waltham Cross and Gordo grapes than in fresh Sultanas.

Discussion

The composition of the surface lipids of the grapes which are mainly used for drying appears to be fairly similar. The triterpene oleanolic acid is the main constituent of the lipids, followed by alcohols, free acids, esters and aldehydes and hydrocarbons. The knowledge of the composition of these natural lipids facilitates the determination of processing aids added to the dried grapes during drying and processing.

It has been shown[12] that the surface wax of Sultana grapes contains considerable amounts of n-aldehydes, which are mainly destroyed during sun drying. Unfortunately the chromatographic behaviour of n-aldehydes, their polymerisation products, and the ethyl esters of fatty acids is very similar, so that thin-layer chromatography did not reveal if the aldehydes of cold dipped Sultanas are destroyed during drying. Hydrolysis of the waxes converts the aldehydes to polymerisation products, which are mainly found in the unsaponifiable fraction.

The saponifiable fraction of the lipids of the varieties Gordo and Waltham Cross contains a much higher amount of the n-C_{16} and n-C_{18} acids than the seedless Sultana grapes. The content of n-C_{16} and n-C_{18} acids in the free acid fraction of surface lipids from several grape varieties was found not to exceed more than 20% of the total fatty acid fraction.[18a] It is therefore likely that some internal lipids of the seeded grape varieties are extracted with chloroform. Grape seed oil contains mainly n-C_{16} and n-C_{18} acids.[19]

The results presented indicate that two methods for the determination of lipid additives appear feasible. The light petroleum-soluble lipids can be separated into the main fractions on aluminium oxide. Any shift from the normal composition of the surface lipids can then easily be

detected and determined. The saponification of the petroleum-soluble lipids is likely to yield the same information. Any addition of oil or fat leads to an increase in the saponifiable fraction. An uptake of about 1 g. of oil per kg. of dried Sultanas has to be regarded as normal and inherent to the method for the production of light coloured Sultanas. It was shown that only very little of this ' dipping oil ' is removed by the commercial washing procedure of dried grapes.

The addition of mineral oil can easily be detected and determined by thin-layer chromatography; saponification of mineral oil containing lipids results in high amounts of unsaponifiable material. If mineral oil is replaced by animal or plant oils, these oils which consist mainly of glycerides would appear on chromatography on aluminium oxide in the ' ester-aldehyde fraction ' or on hydrolysis in the saponifiable part. If an accurate determination and identification of the acids is wanted this can be achieved by gas–liquid chromatography of the methyl esters.

Acknowledgments

The author is greatly indebted to Dr. J. V. Possingham for support of this work and valuable criticism, to Dr. D. H. S. Horn of C.S.I.R.O. Division of Organic Chemistry, Melbourne, for advice and co-operation, to Mr. A. A. Gregory of the Dept. of Primary Industry for supplying samples of dried fruit, and to Mrs. E. Torokfalvy and Mr. M. V. Grncarevic for technical assistance.

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Received 22 February, 1965

References

[1] Winkler, A. J., ' General Viticulture ', 1962, p. 566 (Berkeley and Los Angeles: University of California Press)
[2] Cruess, W. V., ' Commercial Fruit and Vegetable Products ', 1958, 4th edn, p. 540 (New York-Toronto-London: McGraw-Hill Book Co.)
[3] Permezel, N. C., *World Crops*, 1964, (June), p. 22
[4] Radler, F., *J. Sci. Fd Agric.*, 1964, **15**, 864
[5] Martin, R. J. L., & Stott, G. L., *Aust. J. agric. Res.*, 1957, **8**, 444
[6] Dudman, W. F., & Grncarevic, M.V., *J. Sci. Fd Agric.*, 1962, **13**, 221
[7] Radler, F., & Grncarevic, M. V., *Fd Technol. Aust.*, 1964, **16**, 518
[8] Krummel, G., *Dtsch. Lebensm. Rundschau*, 1962, **58**, 36
[9] Krummel, G., *Z. Lebensm.Unters.*, 1963, **117**, 194
[10] Radler, F., & Grncarevic, M. V., *J. agric. Fd Chem.*, 1964, **12**, 266
[11] Werner, H., Wurziger, J., & Ristow, R., *Dtsch. Lebensm. Rundschau*, 1964, **60**, 133
[12] Radler, F., & Horn, D. H. S., *Aust. J. Chem.*, 1965, **18**, 1059
[13] Martin, J. T., *J. Sci. Fd Agric.*, 1960, **11**, 635
[14] Brieskorn, C. H., & Hofmann, H., *Arch. Pharm. Berl.*, 1962, **295**, 505
[15] Matic, M., & Horn, D. H. S., *J. Sci. Fd Agric.*, 1957, **8**, 571
[16] Mangold, H. K., ' Aliphatische Lipide ' in ' Dünnschicht Chromatographie ' (Stahl, H., ed.), 1962, p. 182 (Berlin-Göttingen-Heidelberg: Springer-Verlag)
[17] Burton, D., & Robertshaw, G. F., ' Sulphated Oils and Allied Products ', 1939, p. 93 (London: A. Harvey)
[18] Lamberton, J. A., *Aust. J. Chem.*, 1965, **18**, 911
[18a] Radler, F., *Amer. J. Enol. Vitic.* (in press)
[19] Meara, M. L., ' The Fats of Higher Plants ', in ' Encyclopedia of Plant Physiology ' (ed. Ruhland, W.), 1957, Vol. VII, p. 31 (Berlin-Göttingen-Heidelberg: Springer-Verlag)

ISOLATION AND CHARACTERISATION OF WHEAT FLOUR PROTEINS*

I.—Separation of salt- and acetic acid-dispersible proteins by gel filtration, polyacrylamide gel electrophoresis, and sucrose gradient ultracentrifugation

By M. JANKIEWICZ† and Y. POMERANZ

Proteins extracted from wheat flour with 0·01 M-sodium pyrophosphate buffer, pH 7·0, and with 0·05 M-acetic acid were centrifuged, dialysed against 0·005 M-sodium acetate buffer pH 4·1, concentrated by ultrafiltration at 4°, and lyophylised. The proteins were dispersed in 0·005 M-sodium acetate buffer pH 4·1 and fractionated on Sephadex G-100 and Sephadex G-200 columns. Based on separation according to average mol. wt. on Sephadex G-100, the pyrophosphate-dispersible proteins contained eight, and the following acetic acid-dispersible proteins six fractions. Proteins extracted directly with 0·05 M-acetic acid were separated into seven fractions. The distribution of protein moieties of different average molecular-size ranges is detailed and shown to be different in the different extracts. Aggregation and disaggregation phenomena were observed during repeated re-chromatography of high- and intermediate-molecular weight proteins. Ultra-centrifugation in a stepwise sucrose gradient at 45,000 *g.* at 4° and fractionation by polyacrylamide-gel electrophoresis separated the protein into slow-moving, intermediate- and high-molecular weight moieties. The relative distribution of these moieties was correlated with the mol. wt. of the fractions eluted from Sephadex columns.

Introduction

Despite considerable progress in cereal protein chemistry in such fields as fractionation of proteins,[1–6] amino-acid composition,[7–9] or the role of sulphydryl groups in protein structure,[10–13] many other aspects remain unresolved or require revision.

It is well established[14, 15] that the procedure used to isolate proteins and the methods employed for preliminary fractionation, may have a profound effect on the material investigated. Conformational changes of the proteins in some ranges of pH and ionic strength[16, 17] are among the sources of major differences in protein properties reported in literature. A large number of fractions have been isolated by starch-gel or polyacrylamide-gel electrophoresis.[15] Some fractions are likely to have resulted from interaction between proteins and buffer ions,[18] or from partial dissociation or aggregation of reacting components of the heterogenous protein complex.[15, 16]

Fractionation of proteins on Sephadex molecular sieves has resulted in recent years in an impressive volume of valuable information on proteins from numerous sources. Fractionation techniques on Sephadex are attractive because they are accompanied by rather minimal alterations of the proteins investigated and because the fractionations depend on differences in molecular size and shape. Fractionations on Sephadex columns of cereal proteins have been reported.[19–23]

This study was initiated to evaluate, critically, use of the Sephadex filtration technique as related to cereal proteins; to develop a procedure for large-scale preparations; and to compare the products of molecular-sieve separation by other methods, such as separation by polyacrylamide-gel electrophoresis and sucrose gradient-centrifugation. In all dialysis and fractionation techniques, a monovalent, acetate buffer of low ionic strength was used under conditions which dispersed wheat proteins without use of solubilising agents such as dimethylformamide or urea.

* This work is part of the co-operative investigations of Crops Research Division, Agricultural Research Service, U.S. Dept. of Agriculture and Dept. of Flour & Feed Milling Industries, Kansas Agric. Exp. Sta., Manhattan. Contribution No. 491 from the Kansas Agric. Exp. Sta. A report of work done in part under contract with the U.S. Dept. of Agriculture and authorised by the Research & Marketing Act of 1946. This contract was supervised by the Western Research & Development Div., Agricultural Research Service.

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Experimental

The flour was experimentally milled from a composite grist of several hard winter wheat varieties grown at a number of locations throughout the Great Plains (U.S.A.) in 1963.[24] Extraction of the proteins from the flour, concentration by ultrafiltration, dialysis and fractionation were performed at 4° by means of the buffer systems described by Coates & Simmonds[25] with some modifications. The sample of flour (76·72 g.) containing 10 g. of protein (N × 5·7) was homogenised for 2 min. in a Waring Blendor* with 250 ml. of 0·01 M-sodium pyrophosphate buffer. The suspension was shaken for 1 h., centrifuged on an International centrifuge for 25 min. at 5000 *g*. The residue was returned to the Waring Blendor and the whole procedure was repeated three times. Combined centrifuged extracts were made up to 1000 ml. with the buffer, and the solution set aside at 4° overnight. The solid material was next extracted in an analogous manner with four 250-ml. portions of 0·05 M-acetic acid. In a separate experiment, the flour proteins were extracted directly with 0·05 M-acetic acid without a preliminary extraction with pyrophosphate buffer pH 7·0.

Protein extracts were clarified by centrifuging for 15 min. at 24,000 *g* on a Serval centrifuge. Total protein was determined by a Kjeldahl semimicro procedure.[26] The extracts were concentrated during 72 h. to about 250 ml. by use of an ultrafiltration technique employing a device similar to that described by Siegelman & Firer.[27]

Concentrated extracts were dialysed in a continuous procedure against about 10 l. of 0·005 M-sodium acetate buffer, pH 4·1, for 48 h. Dialysed extracts were concentrated to 100–150 ml. in a small ultrafiltration unit, and lyophylised.

Molecular filtration of the proteins was performed on modified dextrans, Sephadex G-100 and G-200. Calculated amounts of the dextrans were suspended in 0·05 M-sodium chloride solution, and shaken at hourly intervals for 6 h. The suspensions were then poured into 6·0 × 72·0-cm. or 3·0 × 72·0-cm. columns fitted with uniformly prepared powdered glass–sand supports to prevent dead space or turbulence during elution from the column. After complete sedimentation of Sephadex in the columns, the tops of the columns were connected to a reservoir containing 0·005 M-sodium acetate buffer, pH 4·1, and washed for about 72 h. until the eluate was free from chloride ion. For fractionation on the column, 50-ml. aliquots of protein dispersions containing 0·5 or 2·0 g. of protein in the 0·005 M-sodium acetate buffer were placed on top of the column. The flow of the buffer through the column was maintained at 10 ml. per h. and 10-ml. fractions were collected on a fraction collector. Eluates from the columns were examined at 280 mμ and at 610 mμ with a Beckman D.U. Spectrophotometer. (Readings were made at 610 mμ as the eluate was opalescent and colloidal in nature.) Fractions varying in molecular weight collected from the columns showed a different optical density response when scanned in the ultra-violet region by a Cary Model 11 spectrophotometer (Fig. 1). The optical density of the proteins in the pyrophosphate buffer extract is compared with optical density readings of the high-molecular fractions from the acetic-acid extract in Fig. 2. While in elution curves from Sephadex columns, optical density at 280 mμ is recorded, the protein distribution in the various fractions collected from the Sephadex G-100 columns is computed from semimicro Kjeldahl-N determinations. Columns of Sephadex G-100 were calibrated by passing dispersions of purified, crystalline proteins of known mol. wt. according to the methods of Whitaker[28] and of Andrews.[29] [Crystallised bovine plasma albumin was from Armour Laboratories; alcohol dehydrogenase from yeast (twice crystallised), lysozyme (three times crystallised) and pepsin (twice crystallised) were from Mann Research Laboratories; catalase (C grade, lot 32924) and edestin (hempseed, crystallised, C grade, lot 33594) from Calbiochem. Corp.; trypsin, salt free (twice crystallised) from Nutritional Biochemical Corp.; bovine γ-globulin, Fraction II, from Pentex Corp.; and ovalbumin from Worthington Biochemical Corp.] In Fig. 3 is plotted the eluate volume against the log of mol. wt. of the pure proteins. Bovine albumin and ovalbumin contained under conditions of the test, mixtures of monomer and dimer. Edestin was a heterogeneous mixture with main peaks eluted at 620 and 810 ml. Yeast alcohol-dehydrogenase precipitated on the column and could not be eluted under the conditions employed, and catalase

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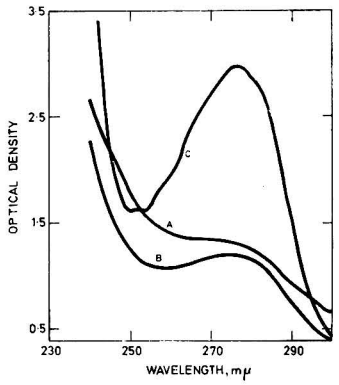

FIG. 1.—*A scan of wheat flour extracts in the ultra-violet region on a Cary Model* 11 *spectrophotometer*

A high mol. wt. protein fraction from acetic acid extract. B intermediate mol. wt. protein fraction from acetic acid extract. C proteins dispersible in pyrophosphate buffer, pH 7·0

behaved anomalously and was eluted much later than expected. At the extreme ends, both lysozyme and γ-globulin were slightly delayed, but over the range of mol. wt. 20,000–150,000, the plot is linear with a reasonable degree of accuracy.

Polyacrylamide-gel electrophoresis was performed on 6 mm. × 75 mm. columns, with a gel containing 7·5% of acrylamide and 0·2% of *NN*-methylenebisacrylamide polymerised according to Barka.[30] The electrolytes were removed electrophoretically for 3 h. from the column at 500 V. A dispersion containing 0·5–1·0 mg. of protein in 10–20 μl. of 36% sucrose (w/v) in acetate buffer pH 4·1 was fractionated for 1 h. at 125 V, followed by 4 h. at 500 V. The gel columns removed from the electrophoresis tubes were stained overnight in test tubes containing 0·1% of Amidoblack B and background stain removed by means of an electric current.[31] Protein fractions, separated on the poly-acrylamide-gel columns, were determined with a densitometer with scanning stage and varicord recorder (Photovolt Corp.).

Ultracentrifugation of the wheat flour protein fractions in sucrose gradient was performed on a preparative Model L Spinco ultracentrifuge using a SW 25 rotor. During preliminary experiments, sucrose gradients in ranges 4–36, 10–30, 4–20, and 3–15% were used as stepwise and continuous gradients (by holding a stepwise gradient overnight at 4°), and by varying centrifugation from 45 min. to 25 h. Best results were obtained by a stepwise sucrose gradient in 0·005 M-sodium acetate buffer pH 4·1. The gradient was formed by carefully layering in the centrifuge tubes of 5-ml. portions of solutions containing 36, 28, 20, 12 and 4% of sucrose (w/v),

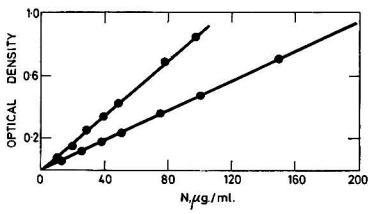

FIG. 2.—*Optical density* (280 mμ, *Beckman DU spectrophotometer*) *of equivalent protein levels from pyrophosphate buffer* (*upper line*) *and acetic acid soluble proteins* (*lower line*)

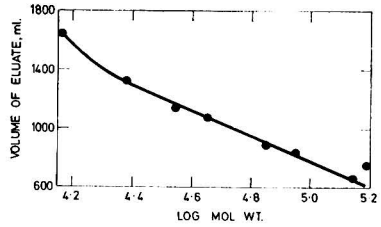

FIG. 3.—*Relationship between elution volume and log of mol. wt. of pure proteins. From left to right: lysozyme, trypsin, pepsin, ovalbumin monomer, bovine plasma albumin monomer, ovalbumin dimer, bovine plasma albumin dimer, and γ-globulin*

respectively. Two-ml. aliquots of protein dispersion in 0·005 M-sodium acetate buffer pH 4·1 were placed on top of the gradient and covered with 1 ml. of the same buffer. Centrifugation was performed for 18 h. at 45,000 *g* at 4°. After centrifugation, the tubes were punctured at the bottom and approximately 0·7-ml. aliquots of solution were collected for spectrophotometric examination at 280 mμ.

Results and discussion

Nitrogen contents of the protein extracts prepared with the different extracting systems were determined and calculated as percentage of total N content of the flour. Results are presented in Table I.

Table I

Nitrogen contents in wheat flour extracts prepared with 0·01 M-*sodium pyrophosphate buffer, pH* 7·0, *and* 0·05 M-*acetic acid*

Extraction	N in extract, expressed as % of total flour N	N in lyophylised sample as % of total flour N
(1) Two-step extraction		
A. 0·01 M-sodium pyrophosphate buffer, pH 7·0	15·2	8·3
B. 0·05 M-acetic acid	76·1	53·1
TOTAL	91·3	61·4
(2) One-step extraction		
0·05 M-acetic acid	89·6	77·5

Over 90% of flour nitrogen was extracted by the two-step procedure with pyrophosphate buffer followed by acetic acid. The ratio of nitrogen extracted by pyrophosphate buffer to acetic acid-dispersed nitrogenous substances was 1 : 5. Extraction with acetic acid alone gave slightly lower yields of dispersed proteins in extracts. Multiple operations of dialysis, two ultracentrifugation steps, and lyophylisation resulted in losses of material. For proteins extracted by the two-step procedure, yields of lyophylised material were reduced by 46% and 30% for the pyrophosphate buffer and the acetic acid extracted proteins, respectively. Higher losses in proteins of low molecular weight from pyrophosphate buffer extracts seem to have resulted by the operations using Cellophane tubing.

Fractionation of the proteins on large Sephadex G-100 columns were performed with 0·5 and 2·0 g. of the pyrophosphate- and acetic acid-dispersible proteins, respectively. Proteins separated on the columns from duplicate fractionations (each fractionation being made on protein extracted separately from the flour) were pooled, concentrated by ultrafiltration, and rechromatographed on the columns.

Fig. 4 illustrates results obtained during fractionation of wheat flour proteins on Sephadex G-100 columns. The distribution of various fractions in the pyrophosphate, acetic acid following pyrophosphate, and acetic acid extracts are given in Table II. Extraction of proteins directly with 0·05 M-acetic acid resulted in a distribution of molecular weight moieties, which differed from the distribution to be expected from computation of the contribution of the pyrophosphate- and acetic acid-dispersible proteins. The proteins extracted directly with 0·05 M-acetic acid contained less or practically none of the low molecular weight proteins (Fractions V–VIII in pyrophosphate-dispersible extract); and the amount of Fractions III and IV was increased at the expense of Fractions I and II. These results would seem to indicate that the low-molecular weight proteins extracted with pyrophosphate buffer are capable of modifying and reacting with the large gluten proteins. The results are in line with the present concept regarding the rôle of salt-extractable proteins in dough structure.

Heterogeneity of acetic acid-dispersible proteins separated on Sephadex G-100 into material under three main peaks was demonstrated by several methods, including: differences in shape of peaks as determined by optical density measurements at 280 and 610 mμ; rechromatography of the material on Sephadex G-100 and on Sephadex G-200; asymmetrical shape of peaks; viscosimetry; polarimetry; refractive index; polyacrylamide gel electrophoresis; and sucrose gradient-ultracentrifugation.

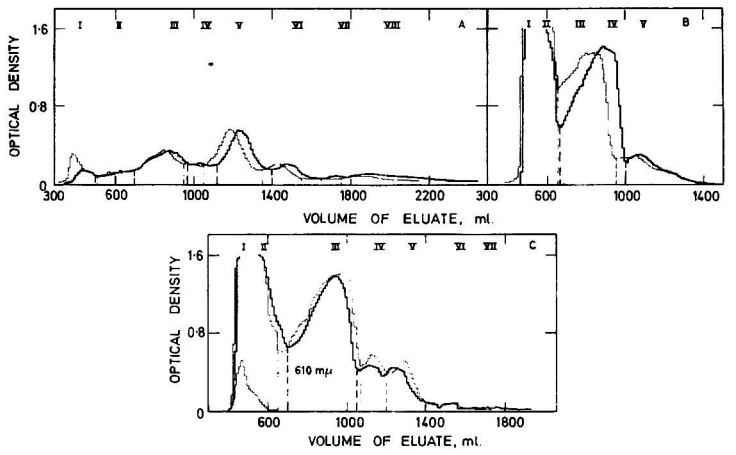

FIG. 4.—*Fractionation of wheat flour proteins on a Sephadex G-100 column, 6·0 × 72·0 cm.*

A 0·5 g. of protein extracted from flour with 0·01 M-sodium pyrophosphate buffer, pH 7·0
B 2·0 g. of protein extracted from flour with 0·05 M-acetic acid following pyrophosphate buffer
C 2·0 g. of protein extracted from flour with 0·05 M-acetic acid
All fractionations were made in duplicate, each from a separate extract of the flour. Eluant: 0·005 M-sodium acetate buffer, pH 4·1; extinctions of 10-ml. fractions measured at 280 mμ in a 1-cm. cuvette

Table II

Distribution of various wheat flour proteins (as % of total) fractionated on Sephadex G-100

Fraction No.	Average mol. wt.	Two-step extraction		One-step extraction
		Pyrophosphate	Acetic acid	Acetic acid
I	not resolved	5·7	47·9	40·4
II	above 150,000	11·8		
III	83,000	16·9	40·0	49·8
IV	53,000	11·3		
V	32,000	34·5	12·1	4·6
VI	18,000	8·8		4·6
VII	about 15,000	3·2	traces	0·6
VIII	below 15,000	7·8	traces	traces

Despite the different fractions that predominated in pyrophosphate and acetic acid extracts, both contained proteins of widely varying molecular weight. Rather exhaustive extraction of flour with the pyrophosphate buffer did not exclude substantial amounts of low molecular-sized fractions (average molecular weight of 32,000 and 18,000) in the acetic acid-soluble dispersions (Fig. 5). Even after double rechromatography of the peak of high-molecular proteins (Fig. 5A, fractions I and II, mol. wt. above 150,000), a substantial amount of fraction III with average molecular weight 83,000 continued to be present in twice-rechromatographed material (Fig. 5C). At least two moieties in the first peak are indicated by differences in the shape of curves obtained during spectrophotometry at 280 and 610 mμ.

The second peak (Fig. 5A, a mixture of fractions II and III with average mol. wt. of above 150,000 and of 83,000) on repeated rechromatography (Fig. 5D–5F), gave asymmetrical elution curves of the main component. The rechromatographed proteins contained substantial amounts of high-molecular weight material comparable to the I and II fractions, as well as low-molecular proteins (Fractions IV and V; average mol. wt. of 53,000 and 32,000, respectively). High-molecular weight proteins in rechromatographed fractions II and III seem to have resulted from aggregation of moieties of intermediate size.

Rechromatography of Fractions I and II (first peak from Fig. 5A) on Sephadex G-200 columns is illustrated in Fig. 6. The mixture of fractions I and II seemed to be separated into

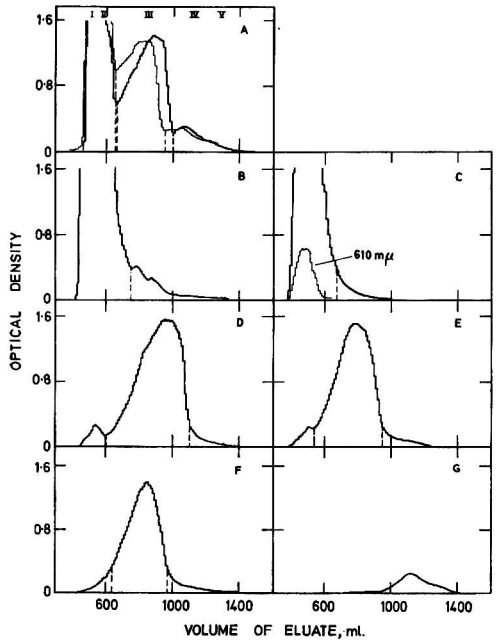

FIG. 5.—*Fractionation of 2·0 g. of protein, extracted from flour with 0·05 M-acetic acid following pyrophosphate buffer, on a Sephadex G-100 column* (6·0 × 72·0 cm.)

Eluant: 0·005 M-sodium acetate buffer, pH 4·1; extinctions of 10-ml. fractions measured at 280 or 610 mμ in a 1-cm. cuvette
A duplicate fractionation of protein from 2 extractions. B-C first and second rechromatography of fractions I-II. D-F first, second and third rechromatography of fractions III-IV (second peak)
G rechromatography of fractions V-VI (third peak)
Eluates used for rechromatography are shown by vertical lines in a previous fractionation step

a fraction of high and intermediate molecular weight by the better resolution power of Sephadex G-200 for high-molecular weight proteins. Shapes of spectrophotometric curves at 280 and 610 mμ for the first peak in Fig. 6 are similar but unlike the shapes in Fig. 5C.

Results of fractionation of wheat flour proteins by electrophoresis on polyacrylamide gel are illustrated in Fig. 7. Whereas the pyrophosphate buffer-dispersible proteins contained

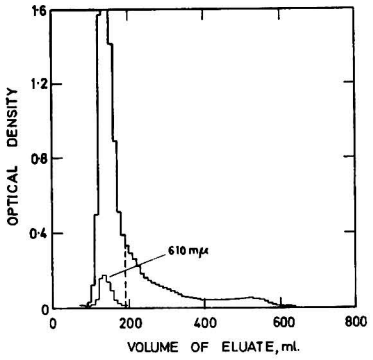

FIG. 6.—*Fractionation of the first peak from Fig. 5A (mixture of fractions I-II) on Sephadex G-200 column* (3·0 × 72·0 *cm.*)

Eluant: 0·005 M-sodium acetate buffer, pH 4·1; extinctions of 10-ml. fractions measured at 280 mμ in 1-cm. cuvette

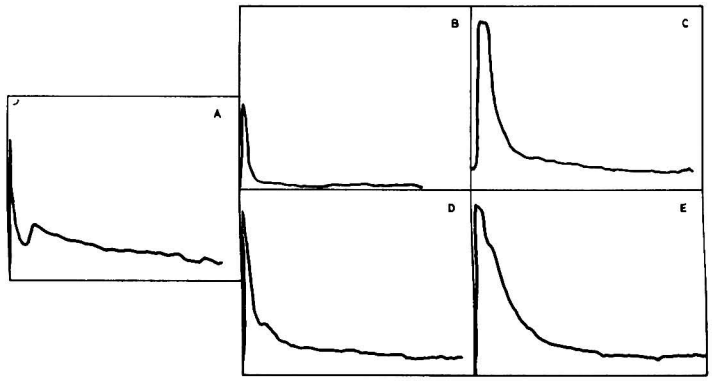

FIG. 7.—*Densitometric determination of stained protein fractions separated electrophoretically on polyacrylamide-gel columns*

A 0·5 mg. of 0·01 M-sodium pyrophosphate buffer-soluble protein; B and C 0·5 and 1·0 mg. of protein extracted with 0·05 M-acetic acid following A; D and E 0·5 and 1·0 mg. of protein extracted directly with acetic acid

mainly rapidly moving components, the proteins extracted with acetic acid, following pyrophosphate extraction, remained almost completely at the point of application. Extraction of the flour directly with 0·05 M-acetic acid yielded a mixture of slow- and fast-moving proteins.

Ultracentrifugation of the extracted flour proteins (Fig. 8) gave a complex character similar to that from fractionation on Sephadex or polyacrylamide-gel electrophoresis. Most protein extracted by pyrophosphate buffer remained in the upper part of the centrifugation tube and only small amounts moved into the concentrated sucrose gradient (Fig. 8A). Protein extracted by acetic acid (following pyrophosphate extraction) contained large quantities of components of high molecular weight deposited at the bottom of centrifuge tubes. Substantial amounts of proteins that moved only a small distance into the gradient, and equilibrated with low-density sucrose gradient-portions also were present in the acetic acid extract (Fig. 8B). The fact that a continuous spectrum of protein moieties was present throughout the sucrose gradient indicates the presence of a highly complex mixture. Results summarised in Fig. 9 were obtained by sucrose gradient-centrifugation of aliquots from the first 50 ml. (A) and last 50 ml. of eluate (B) of fractions I and II (Fig. 5C), and from the middle portions (C) of fractions III and IV (Fig. 5E).

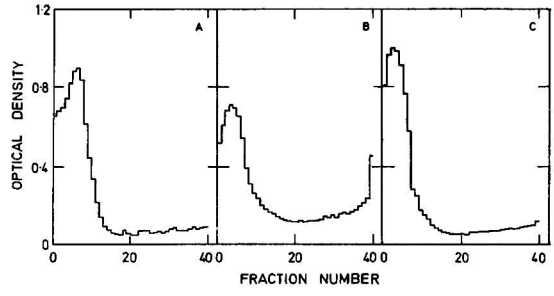

FIG. 8.—*Ultracentrifugation of proteins extracted with* 0·01 M-*sodium pyrophosphate buffer, pH* 4·1 (A), *followed by* 0·05 M-*acetic acid* (B), *and* 0·05 M-*acetic acid* (C)

Spinco preparative ultracentrifuge, Model L, rotor SW-25; 45,000 *g*., 18 h. at 4°. Fractionation of 10 mg. of protein in 2 ml. of buffer in 5 × 5 ml. stepwise sucrose gradients of 36, 28, 20, 12 and 4% (w/v) in 0·005 M-sodium acetate buffer, pH 4·1. Numbering of fractions has been reversed to represent their actual position from the top (low no.) to the bottom (high no.) of the ultracentrifugation tube

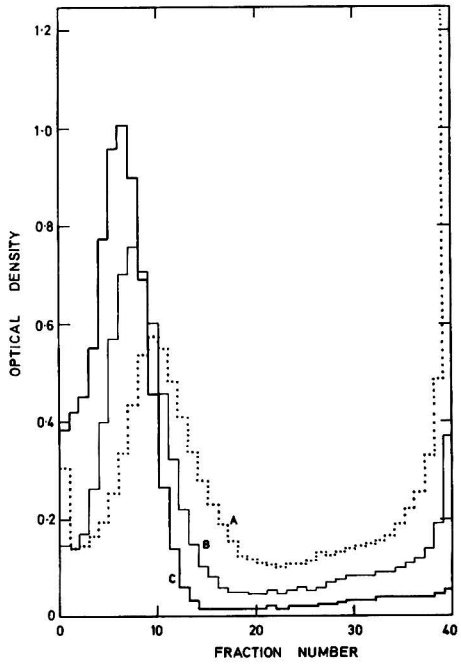

FIG. 9.—*Ultracentrifugation of high- and intermediate-mol. wt. protein fractions*

A aliquot of first 50 ml. of eluate of fractions I–II (Fig. 5C); B aliquot of last 50 ml. of eluate of fractions I–II (Fig. 5C); C aliquot of middle peak portion of fractions III–IV (Fig. 5E)
Experimental conditions as for Fig. 8. Numbering of fractions has been reversed to represent their actual position from the top (low no.) to the bottom (high no.) of the ultracentrifugation tube

The first peak in the ultracentrifuge tube progressively changed from fraction no. 11 to 8 to 7 for samples A, B and C respectively. Fractions of intermediate mobility were highest in A and lowest in C; and the amount of protein deposited on the bottom was again highest in A and lowest in C.

Conclusion

The results of this study indicate that the previous notion of classifying proteins according to solubility criteria alone is unsatisfactory. It implies that wheat proteins, interlinked in a great number of ways, can exist in several conformations, and that the life-length of a certain form depends on conditions of isolation, fractionation and storage. The study of some of these linkages and their effects on the size and overall properties of wheat proteins is the subject of another report.[32]

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Received 24 March, 1965; amended manuscript 8 June, 1965

References

1 Elton, G. A. H., & Ewart, J. A. D., *J. Sci. Fd Agric.*, 1962, **13**, 62
2 Jones, R. W., Taylor, N. W., & Senti, F. R., *Arch. Biochem. Biophys.*, 1959, **84**, 363
3 Lee, J. W., Tracey, M. V., Winzor, D. J., Wrigley, C. W., & Zentner, H., *Aust. J. biol. Sci.*, 1963, **16**, 712
4 Pence, J. W., Weinstein, N. E., & Mecham, D. K., *Cereal Chem.*, 1954, **31**, 396
5 Simmonds, D. H., & Winzor, D. J., *Aust. J. biol. Sci.*, 1961, **14**, 690
6 Woychik, J. H., Boundy, J. A., & Dimler, R. J., *Arch. Biochem. Biophys.*, 1961, **94**, 477
7 Hepburn, F. W., Lewis, E. W., jun., & Elvehjem, C. A., *Cereal Chem.*, 1957, **34**, 312
8 Simmonds, D. H., *Cereal Chem.*, 1962, **39**, 445
9 Woychik, J. H., Boundy, J. A., & Dimler, R. J., *J. agric. Fd Chem.*, 1961, **9**, 307
10 Axford, D. W. E., Campbell, J. D., & Elton, G. A. H., *J. Sci. Fd Agric.*, 1964, **13**, 73
11 Beckwith, A. C., Wall, J. S., & Dimler, R. J., *Fed. Proc.*, 1963, **21**, 1122
12 Frater, A., Hird, F. J. R., Moss, J., & Yates, J. R. *Nature, Lond.*, 1960, **186**, 451
13 Sokol, H. A., Mecham, D. K., & Pence, J. W., *Cereal Chem.*, 1960, **37**, 151
14 Haurowitz, F., 'The Chemistry and Function of Proteins', 1963 (New York: Academic Press)
15 Pence, J. W., Nimmo, C. C., & Hepburn, F. N., 'Proteins', in 'Wheat, Chemistry and Technology' (Ed. I. Hlynka), 1964 (St. Paul, Minn.: Amer. Ass. of Cereal Chemists)
16 Reithel, F. J., *Adv. Protein Chem.*, 1963, **18**, 124
17 Scheraga, H., 'Protein Structure', 1961 (New York: Academic Press)
18 Cann, J. R., *J. Amer. chem. Soc.*, 1958, **80**, 4263
19 Abbott, D. C., 1964, Ph.D. Thesis, Kansas State University
20 Djurtoft, R., *Wallerstein Labs Commun.*, 1963, **26**, 83
21 Nummi, M., *Acta chem. scand.*, 1963, **17**, 527
22 Wright, W. B., Brown, P. J., & Bell, A. V., *J. Sci. Fd Agric.*, 1964, **15**, 56
23 Jones, R. W., Babcock, G. E., Taylor, N. W., & Dimler, R. J., *Cereal Chem.*, 1963, **40**, 409
24 Jankiewicz, M., & Pomeranz, Y., *Cereal Chem.*, 1965, **42**, 37
25 Coates, J. H., & Simmonds, D. H., *Cereal Chem.*, 1961, **38**, 256
26 'Cereal Laboratory Methods', 1962 (St. Paul, Minn.: Amer. Ass. of Cereal Chemists)
27 Siegelman, H. W., & Firer, E. M., *Analyt. Biochem.*, 1962, **3**, 435
28 Whitaker, J. R., *Analyt. Chem.*, 1963, **35**, 1950
29 Andrews, P., *Biochem. J.*, 1964, **91**, 222
30 Barka, T., *J. Histochem. Cytochem.*, 1961, **9**, 542
31 Ferris, T. G., Easterling, R. E., & Budd, R. E., 1963, *J. clin. Pathol.*, **39**, 193
32 Jankiewicz, M., & Pomeranz, Y., *J. Sci. Fd Agric.*, 1965, **16**, 652

ISOLATION AND CHARACTERISATION OF WHEAT FLOUR PROTEINS*

II.†.—Effects of urea and *N*-ethylmaleimide on the behaviour of wheat proteins during extraction and fractionation

By M. JANKIEWICZ** and Y. POMERANZ

Proteins were extracted from wheat flour with seven different extractants. Extraction with 3·0 M-urea at pH 7·0 and at 4° solubilised practically all the proteinaceous material in wheat flour. Extracted proteins were dialysed against 0·005 M-acetate buffer pH 4·1, concentrated by ultrafiltration and lyophylised. Proteins were fractionated in acetate buffer pH 4·1 by sucrose gradient ultracentrifugation or on Sephadex G-100, and the viscosity of Sephadex G-100 eluates was determined. Adding *N*-ethylmaleimide alone, but not in the presence of urea, reduced the yields of proteins extracted in a two-step procedure and increased the relative amounts of proteins of high molecular weight. Adding urea resulted in distinct separation of intermediate-sized proteins into two fractions. Proteins were fractionated on Sephadex G-100 into up to eight fractions, varying in molecular weight, and they were rechromatographed on Sephadex G-100 or G-200. The results indicated aggregation and disaggregation phenomena, and heterogeneity of protein fractions under conditions used.

* Co-operative investigations of Crops Research Division, Agricultural Research Service, U.S. Department of Agriculture and Dept. of Flour & Feed Milling Industries, Kansas Agr. Exp. Sta., Manhattan. Contribution No. 496, Kansas Agricultural Experiment Station, Manhattan. A report of work done in part under contract with the U.S. Department of Agriculture and authorised by the Research and Marketing Act of 1946. This contract was supervised by the Western Research and Development Div., Agricultural Research Service.

† Part I: preceding paper.

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Introduction

Important questions in the chemistry of wheat proteins in general, and of high molecular weight wheat gluten in particular, concern the type of bonds that hold polypeptide strands together and the nature of intermolecular crosslinks responsible for gluten structure. Considerable progress is being made in discovering and elucidating the nature of probable crosslinks in a variety of biological systems. This has resulted in an impressive accumulation of information and contributes to our understanding of physical properties of biological systems, and effects of hydration, mechanical treatment and oxidising agents on the systems.

It is apparent that only a start has been made in understanding the role of various crosslinks in wheat proteins, and in correlating rheological properties of wheat dough with the structure of gluten proteins. The role of sulphydryl and disulphide groups in forming gluten structure and the hypothesis of the reacting systems have been described.[1, 2] The reactivity of sulphur-containing groups of wheat proteins explains, however, only some features of dough rheology. Nearly one-fourth of wheat nitrogen is amide nitrogen, glutamine accounting for most of the amide content.[3] Esterification of side-chain amide groups of gluten and its fractions affects solubility, viscosity and cohesion.[4] We have recently compared effects of *N*-ethylmaleimide and urea on rheological properties of wheat dough, and have found that, in the presence of 3 M-urea, dough structure was completely and almost instantaneously destroyed.[5] Methods to isolate and fractionate wheat flour proteins are described in another paper.[6] This present study was to evaluate the effects of urea and *N*-ethylmaleimide on the isolation and on characteristics of wheat flour proteins, as well as to correlate these effects with gluten properties.

Experimental

The flour, protein extraction procedure, dialysis, ultrafiltration and sucrose-gradient ultracentrifugation, were the same as described previously.[6] In addition to 0·01 M-sodium pyrophosphate buffer pH 7·0, 0·05 M-acetic acid and also 0·05 M-acetic acid following pyrophosphate, flour protein extracts were prepared in the presence of 0·0002 M-*N*-ethylmaleimide (NEMI), 3·0 M-urea, and combinations of these two. NEMI was added to 0·01 M-sodium pyrophosphate buffer pH 7·0. Urea, in 0·01 M sodium pyrophosphate buffer pH 7·0, was added in various concentrations, to solubilise wheat flour proteins. Concentrations of urea above 3·0 M solubilised part of the carbohydrates; below 2·0 M low yields of protein were obtained. Best results were obtained with 3·0 M-urea, which was used in all subsequent tests. Adding 3·0 M-urea in the presence of 0·01 M-sodium pyrophosphate buffer pH 7·0 solubilised most of the extractable protein, and practically no additional protein was extracted by subsequent extraction with 0·05 M-acetic acid.

Results and discussion

The effects of adding urea and *N*-ethylmaleimide on extraction of flour proteins are summarised in Table I. The 3·0 M-urea extract still contained (after extensive ultrafiltration,

Table I

Effects of adding urea and N-ethylmaleimide on extraction of flour proteins

Extractant	Yield of N in extract expressed as % of total flour N	Yield of N in lyophilised sample as % of total flour N	Protein (N × 5·7) in lyophilised material (on dry matter basis), %
A. 0·01 M-sodium pyrophosphate	15·2	8·3	60·0
B. 0·05 M-acetic acid following A	76·1	53·1	85·4
C. 0·05 M-acetic acid	89·6	77·5	97·2
D. 3·0 M-urea in A	—	103·0*	103·1*
E. 0·0002 M-NEMI in A	13·5	6·0	60·0
F. 0·05 M-acetic acid following E	70·6	47·8	93·8
G. 3·0 M-urea and 0·0002 M-NEMI in A	—	64·9	92·1

* The abnormally high yield resulted from the presence of traces of urea in the extracted, dialysed and lyophilised material

dialysis and lyophilisation) traces of urea. Adding NEMI reduced the yield of proteins extracted in a two-step procedure from 91·3 to 83·9%. As this reduction was accompanied by aggregation, it appears that adding NEMI results in partial insolubilisation of the proteins in the extracting media employed.

Data from fractionation of wheat flour proteins from the seven extractants, on Sephadex G-100 are shown in Fig. 1. All proteins were dialysed against 0·005 M-sodium acetate buffer

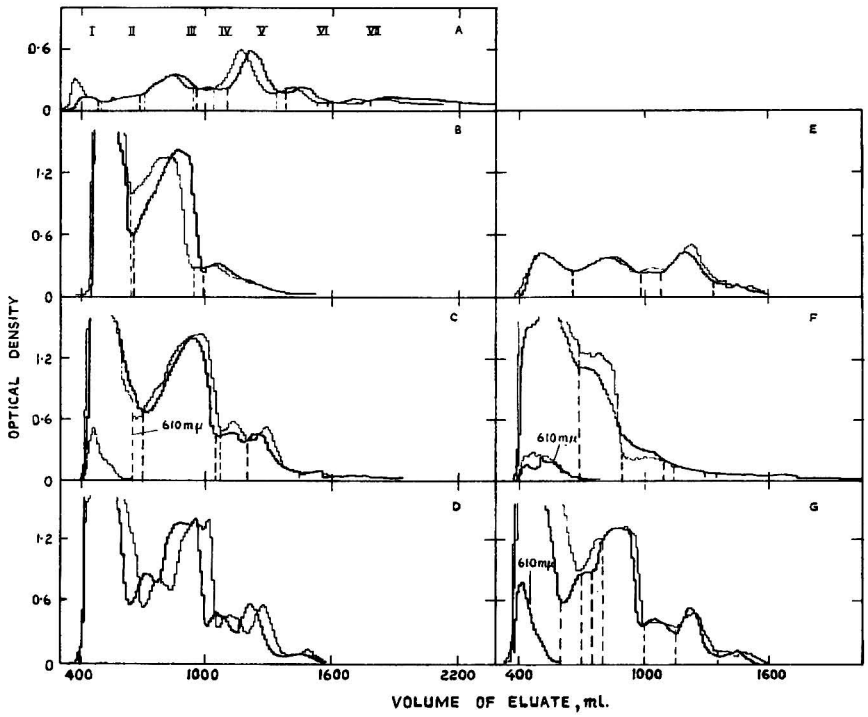

FIG. 1.—*Fractionation of wheat flour protein obtained with various extracting media*

A 0·01 M-sodium pyrophosphate pH 7·0; B 0·05 M-acetic acid following A; C 0·05 M-acetic acid; D 3·0 M-urea in A; E 0·0002 M-NEMI in A; F 0·05 M-acetic acid following E; and G 3·0 M-urea and 0·0002 M-NEMI in A.
Duplicate fractionation of 0·5–2·0 g. of protein on Sephadex G-100 columns (6·0 × 72·0 cm.). The eluant used was 0·005 M-sodium acetate buffer, pH 4·1; extinctions of 10-ml. fractions were measured at 280 mμ or 610 mμ in a 1-cm. cuvette

pH 4·1 before lyophilisation, and the same buffer was used for elution from the column. Distribution of protein fractions varying in molecular weight (according to separation on Sephadex G-100 columns) in proteins from the seven extracting media, is summarised in Table II. Fraction denotations and average molecular weight results are from calculations based on the distribution of proteins soluble in 0·1 M-pyrophosphate buffer, pH 7·0. Slight drifts in average molecular weight were observed in corresponding fractions of chromatographed material extracted with urea and NEMI. The comparison shows that addition of NEMI increased the relative amounts of proteins of high molecular weight, both in the 0·01 M-sodium pyrophosphate buffer pH 7·0, and in the 0·05 M-acetic acid-soluble proteins. The effect of NEMI seems to result from its blocking free thiol groups, and preventing cleavage of inter- and/or intra-molecular disulphide bonds by sulphydryl–disulphide interchange. Adding 3·0 M-urea to the extracting medium, both in the presence and in the absence of 0·0002 M-NEMI, resulted in a distinct separation of the second peak into fractions III and IV which persisted even after urea was removed during dialysis.

Proteins extracted by acetic acid (both directly and after pyrophosphate buffer, pH 7·0) and urea-extracted proteins showed at 610 mμ a slightly skewed curve centred around elution

Table II

Distribution of various wheat protein fractions (as % of total) separated on Sephadex G-100

Fraction No.	Average mol. wt.	Extraction medium* A	B	C	D	E	F	G
I	not resolved	5·7	47·9	40·4	43·1	21·5	51·5	37·1
II	>150,000	11·8						
III	83,000	16·9	40·1	49·8	16·2	34·5	32·2	19·4
IV	53,000	11·3			31·6	8·6	11·0	34·1
V	32,000	34·5	12·1	4·6	3·9	21·5	3·5	4·1
VI	18,000	8·8		4·6	3·4	12·9	1·2	3·5
VII	~15,000	3·2	traces	0·6	1·8	traces	0·6	1·8
VIII	<15,000	7·8	traces	traces	traces	traces	traces	traces

* Extraction media denotations as in Table I

volume of 470 ml. Extraction with acetic acid in the presence of NEMI resulted in a more symmetrical curve (of optical density at 610 mμ), centred around elution volume of 550 ml. The effect of NEMI on the shape of elution curves of total proteins (optical density at 280 mμ) and of turbid protein dispersions (optical density at 610 mμ) was partly cancelled when the proteins were extracted with NEMI in the presence of urea. No working hypothesis can be offered now to explain why adding NEMI in the presence of urea had no significant measurable aggregating effect observed during extraction of wheat flour proteins with NEMI alone. The role of hydrogen bonding in modifying the effects of sulphydryl–disulphide bonds by NEMI, however, suggests a still inexplicable inter-related system.

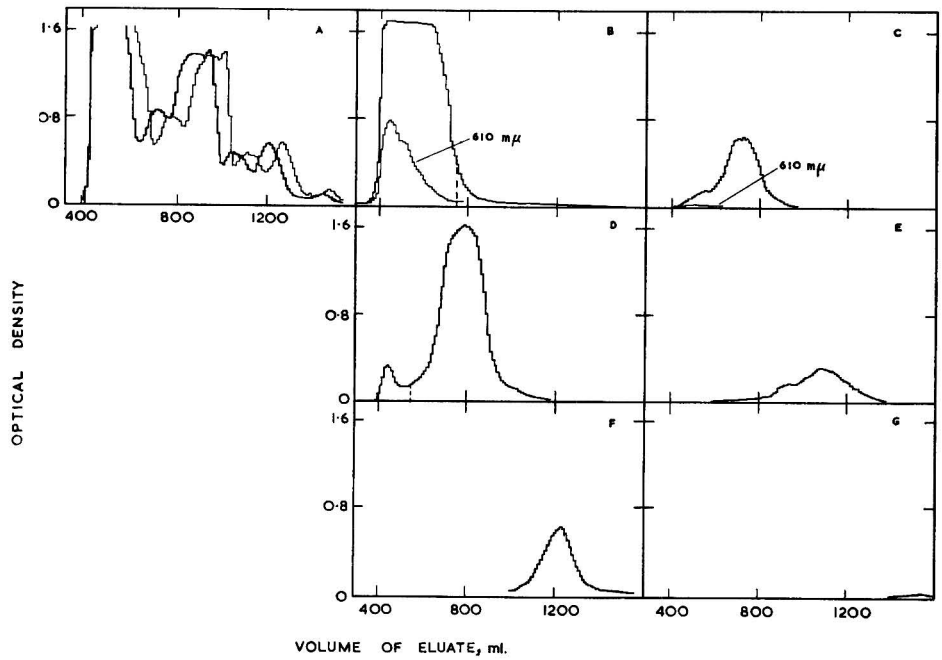

FIG. 2.—*Rechromatography of fractions from urea-extracted proteins*

A. Duplicate fractionation of urea-extracted proteins
B. Rechromatography of fraction I-II (first peak)
C. Rechromatography of fractions III-IV (second peak)
D. Rechromatography of fraction V (third peak)
E. Rechromatography of fraction VI (fourth peak)
F. Rechromatography of fraction VII (fifth peak)
G. Rechromatography of fraction VIII (sixth peak)

The eluant used was 0·005 M-sodium acetate buffer pH 4·1; extinctions of 10-ml. fractions measured at 280 and 610 mμ in a 1-cm. cuvette

Heterogeneity of the various fractions separated by chromatography on Sephadex G-100 is illustrated in Fig. 2, which gives results of rechromatography of six peaks from the urea-extracted material. For rechromatography on Sephadex G-100, pooled eluates from two fractionations of urea-solublised material were concentrated by ultrafiltration. A fraction (2/15) of the eluate under the first peak of urea-soluble material in Fig. 1D was rechromatographed on Sephadex G-200 as shown in Fig. 3. Rechromatography of proteins in this and a previous study seems to point to aggregation and disaggregation phenomena. Thus, material eluted under peak 3 (in

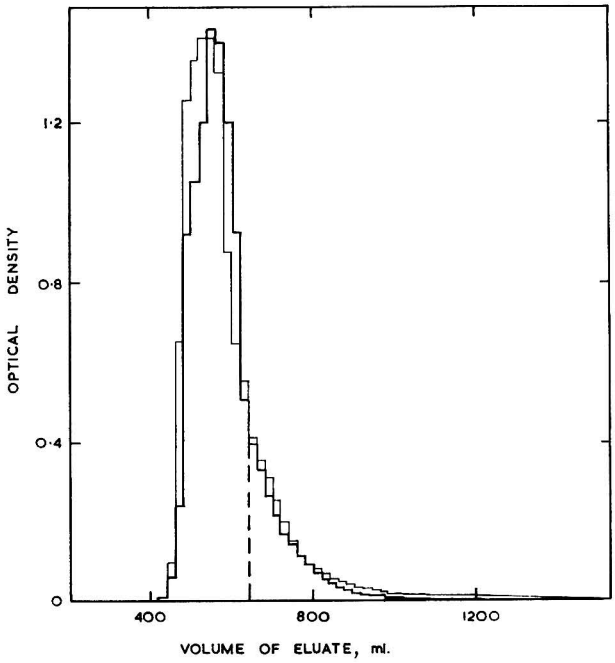

FIG. 3.—*Rechromatography of a portion (2/15) of the first peak of urea-soluble material* (Fig. 1D) *on a Sephadex G-200 column* (3·0 × 72·0 *cm.*)
The eluant used was 0·005 M-sodium acetate buffer pH 4·1; extinctions of 10-ml. fractions measured at 280 mμ in a 1-cm. cuvette

Fig. 1D) (average molecular weight centred around 83,000) was separated during rechromatography into two peaks containing at least three fractions ranging in molecular weight between above 150,000 and 32,000 (in Fig. 2C). Similar phenomena were observed during rechromatography of other fractions. Viscosity determinations of 50-ml. portions of urea-soluble proteins fractionated on Sephadex G-100 (from Fig. 1D) are summarised in Fig. 4. The results show that the first peak of the material eluted contains at least two fractions, one of high and one of low viscosity. The shape and width of the highly viscous eluate corresponds to the shape and width of the turbid protein eluate (Fig. 2A), as determined by optical density measurements at 610 mμ, or the major high-molecular portions separated by rechromatography on Sephadex G-200 (Fig. 3).

Results of sucrose-gradient centrifugation of proteins, extracted from the flour by the seven extracting media, are summarised in Fig. 5. The results confirm those obtained by fractionation on Sephadex G-100. The bulk of the proteins soluble in sodium pyrophosphate buffer pH 7·0 moved a short distance in the ultracentrifugation tube; the amount of proteins of intermediate size was small; and very little material moved to the bottom of the tube. The presence of NEMI in the pyrophosphate extracting medium increased slightly the amount of moieties of

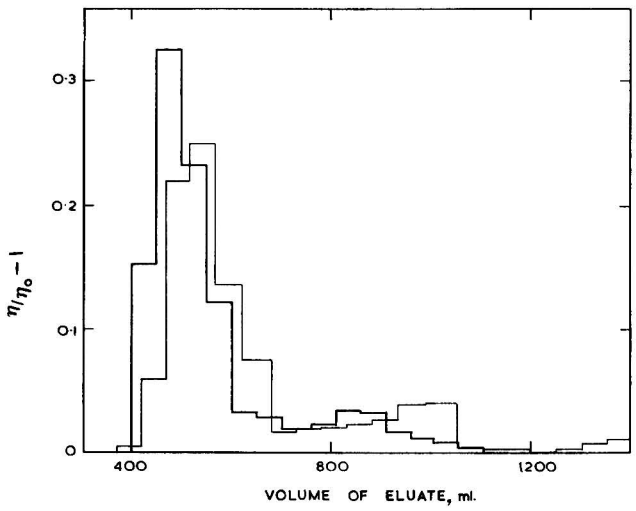

FIG. 4.—*Viscosity determinations of 50-ml. portions of eluates from chromatography of urea-soluble proteins on Sephadex G-100* (Fig. 1D)

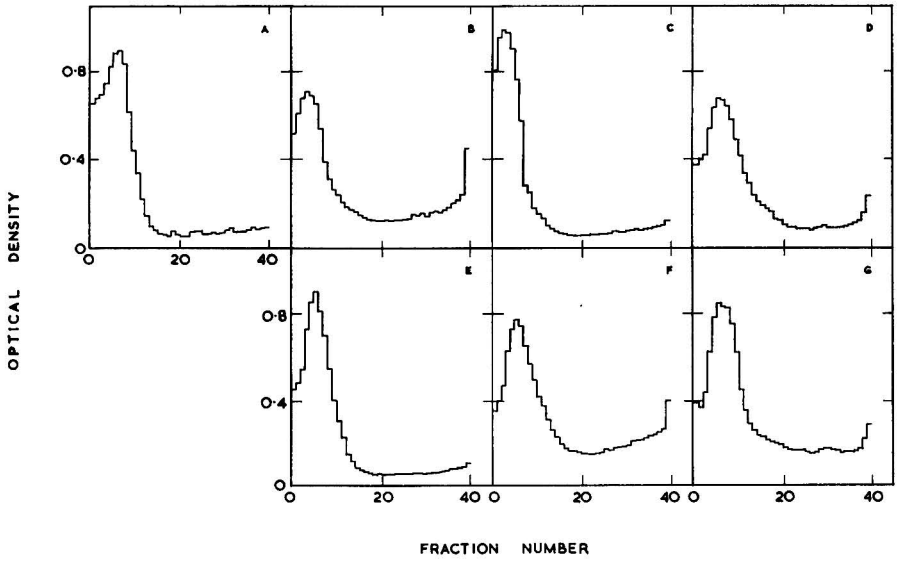

FIG. 5.—*Sucrose-gradient ultracentrifugations of wheat flour proteins in a Spinco preparative ultracentrifuge, Model L, rotor SW-25, at 45,000 g for 18 h. at 4°*

Average results of duplicate fractionation of 10 mg. of protein in 2 ml. of buffer in 5 × 5 ml. stepwise sucrose gradient of 36, 28, 20, 12 and 4% (w/v) in 0·005 M-acetate buffer, pH 4·1

A–G as in Fig. 1. Numbering of fractions has been reversed to represent their actual position from the top (low no.) to the bottom (high no.) of the ultracentrifugation tube

All proteins were dialysed against 0·005 M-sodium acetate buffer pH 4·1 before lyophylisation

high molecular weight and decreased somewhat the amount of slowly running moieties. A similar phenomenon was observed in the acetic acid-soluble proteins (after pyrophosphate treatment). Proteins extracted with urea–pyrophosphate buffer pH 7·0, contained a mixture of slow-moving, intermediate and large-sized proteins. Adding NEMI to the urea–pyrophosphate buffer increased substantially the amount of intermediate-sized protein moieties and proteins that deposited at the bottom of the ultra-centrifuge tube; it also enhanced the penetration of slowly moving proteins into the sucrose gradient.

Conclusions

The phenomena observed indicate the importance of controlling conditions of extracting a heterogeneous mixture of proteins from plant material. Hydrogen-bonding and reactivity of free thiol and of disulphide groups, are among the factors affecting the relative distribution and average molecular weight of plant proteins isolated from the source, and fractionated by molecular sieves or other fractionating systems. The effect of these factors is not cumulative; the resulting modification depends on the relative activity or interaction of factors in a studied system. Extracting proteins with 3·0 M-urea seems of particular interest because the extractant can be used at pH 7·0 at 4°. The use of this extractant is being studied in connection with the isolation of other plant proteins.

Received 26 March, 1965; amended manuscript 8 June, 1965

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References

[1] Pence, J. W., Nimmo, C. C., & Hepburn, F. N., 'Proteins' in 'Wheat Chemistry and Technology' (Ed. I. Hlynka), 1964 (St. Paul, Minn.: American Association of Cereal Chemists)
[2] Villegas, E., Pomeranz, Y., & Shellenberger, J. A., *Cereal Chem*, 1963, **40**, 694
[3] Lin, F. M., Pomeranz, Y., & Shellenberger, J. A., *Proc. 6th Int. Congr. Biochem.*, 1964, II, 117
[4] Beckwith, A. C., Wall, J. S., & Dimler, R. J., *Arch. Biochem. Biophys.* 1963, **103**, 319
[5] Jankiewicz, M. & Pomeranz, Y., *Cereal Chem.*, 1965, **42**, 37
[6] Jankiewicz, M. & Pomeranz, Y., 1965, *J. Sci. Fd Agric.*, 1965, **16**, 644

PHYSICAL ASPECTS OF THE DRYING OF GROUNDNUTS

By O. MYKLESTAD

Investigations have been carried out to establish an economic method for drying unshelled groundnuts to a safe storage moisture content of 9–12% on dry weight in a typically subtropical environment (Queensland). Sorption isotherms on groundnuts showed that to dry the nuts satisfactorily without heat, the ambient air should have R.H. below 75%; but as this condition occurs in the area on the average for only 63% of the time during the harvest season, techniques involving preliminary drying by natural curing in windrows followed by hot-air drying were studied. In a pilot-scale dryer of 40 cu. ft. capacity, an air flow of about 21 cu. ft./(min.) (cu. ft. load) at an air temperature of 100°F satisfactorily reduced the moisture content from 31% to 12% (dry basis) in 24 h. A larger industrial bin dryer of 750 cu. ft. capacity gave even more satisfactory results. Fuel consumption for the larger unit was 2·2 gal./h. at a fuel cost of 24d. (Australian)/gal., and a load of about 7 tons (wet) could therefore be dried at a fuel cost of about 0·12d. (Aust.)/lb. product. The results might be applicable to the drying of groundnuts in other subtropical areas, and the fundamental principles involved might prove applicable to the drying of farm crops in general.

Introduction

Research into the agronomy, chemistry and technology of groundnuts has been carried out for more than 130 years and has led to an extensive literature,[1-3] in which improvements in

drying techniques have received considerable attention. Although originally a tropical plant, the groundnut is extensively grown in subtropical countries throughout the world. About 15,000 tons of the crop is harvested annually in Australia,[4] whose main centre of production is Kingaroy, Queensland. As this is a typically subtropical area, solutions to some of the drying problems facing the Australian groundnut industry there might well be applicable to large subtropical areas of other continents also.

For some years Australian groundnuts have mainly been dried in windrows, but in spite of its adaptability to mechanisation, this method has a number of weaknesses. Firstly, nut losses due to rotting in the field are high, particularly during rainy harvests. Secondly, fungal discoloration of nut shells is often severe (dark brown to black), and growth of a toxin-producing strain of *Aspergillus flavus* is possible.[5,6] Thirdly, off-flavours may develop in nuts directly exposed to the sun. These difficulties may to a certain extent be overcome by artificial drying, many methods of which have been tried during the last 20 years.[7]

Artificially dried groundnuts of high quality can only be produced if certain control requirements are observed. It is essential that unshelled groundnuts be dried to a safe storage moisture content* of about 9–12%.[8] Drying beyond this point is not only unnecessary and costly, but also eventually results in a brittle product which easily splits and cracks. Instances of bagged groundnuts becoming overdry at the air entrance of the dryer have been reported by Sorenson,[9] who observed kernel splitting at moisture contents below 7%. Baker *et al.*[10] noted similar tendencies to overdrying in bulk apparatus, and their attempts to produce evenly dried nuts by a continuous process were only partially successful.

It is generally agreed that over-heating groundnuts (i.e. beyond 120°F) during curing and drying results in off-flavours and impaired germination.[11] Taste tests have indicated that the most palatable nuts are those dried at 85–90°F.[10]

The work reported in the present paper includes a statistical evaluation of weather conditions, measurements of water sorption isotherms for the whole groundnut and for its composite parts, pilot plant experiments to establish suitable artificial drying conditions for whole nuts and, finally, the testing of a moderately sized industrial dryer designed specially for local conditions.

Experimental

Meteorological data

Meteorological data for the last 15 harvesting seasons in the Kingaroy district of Queensland, Australia, were obtained from records of the Queensland Weather Bureau.

Raw material

Raw material used for the laboratory experiments and for the drying tests was Virginia Bunch groundnuts, grown in Kingaroy, Queensland.

Sorption isotherms

Sorption isotherms were determined at 15 and 25°C (59 and 77°F) by keeping groundnut samples in jars with atmospheres of known relative humidities (R.H.) until sample weights were constant. This took about two weeks when the moisture of the nuts was initially 9%. Air humidities were maintained constant by specified salt solutions,[12,13] and moisture analyses of the groundnut samples were carried out gravimetrically.[14]

Equipment for pilot plant studies

During the harvest of 1963 a number of small-scale drying experiments were carried out at Kingaroy, Queensland. Various dryer designs were tested, and the apparatus of one of the most instructive experiments is shown in Fig. 1. In the batch-operated bin (A) 40 cu. ft. of groundnuts were dried with air blown by fan (F) and heated by electrical elements (E). The air flow was measured with a pitot tube at (G), the point of entry of atmospheric air. The temperature of the heated air was measured with thermometers in four symmetrically placed tubes (C).

*All moisture contents given are on a dry-weight basis unless otherwise specified.

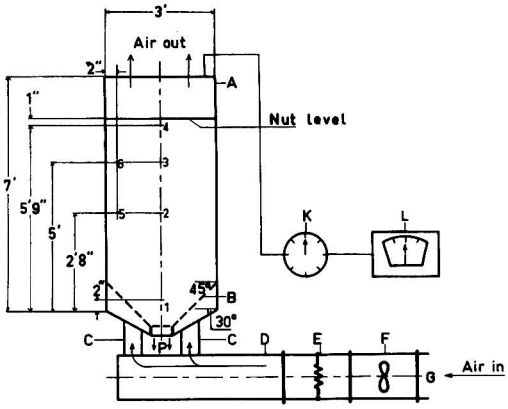

FIG. 1.—*Pilot dryer and auxiliaries*

Temperatures in the load were measured by means of copper–constantan thermocouples connected to a potentiometer (L) through the selector switch (K). Some thermocouples were situated in air surrounding the groundnuts, while others were embedded in the flesh of selected kernels. Only measurements in positions (1) to (4) will be included in this paper.

Groundnut samples were collected from positions at (4), (5) and (6) and their moisture contents determined gravimetrically.[14]

Semi-industrial scale bin dryer

The design and operation of the batch-operated bin dryer shown in Fig. 2 were based on the pilot plant studies. The 800 cu. ft. mild steel bin (A) was filled to a height of 7 ft. 6 in. with groundnuts supported on the perforated floor (B). The nuts, which had been naturally cured in windrows for 5 days, entered the dryer at an average moisture of about 30%. Warm air was supplied by an aerator (E), which was a combination of a diesel-operated fan and an indirect heat exchanger. The aerator was operated to deliver 14,000 cu. ft. of air per minute, which corres-

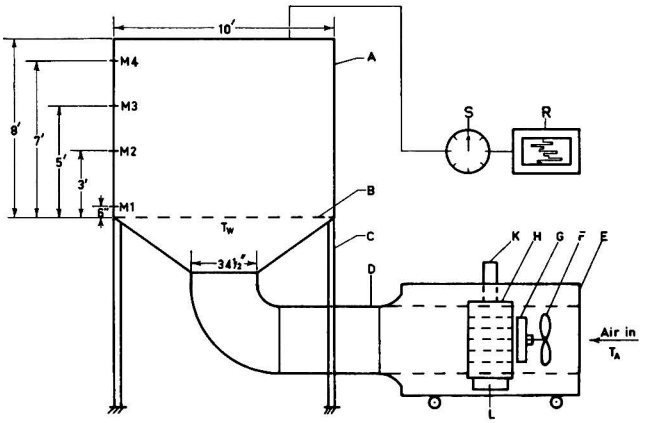

FIG. 2.—*Farm equipment for bulk drying of groundnuts*

ponds to a specific flow of about 19 cu. ft. air/(min.) (cu. ft. load). Temperatures of the atmosphere and of various locations within the load were measured with copper–constantan thermocouples and recorded at (R). Groundnut samples to be analysed were collected from positions (M_1)–(M_4) at regular intervals during tests carried out in the 1964 harvest at Wooroolin, Queensland.

Results and Discussion

Meteorological analysis

Rainfall.—Fig. 3 shows the cumulative rainfall probability, based on the previous 15 years, for Kingaroy, Queensland, during the harvesting season (April–June). The curve indicates that on the average there was no rain for 79% of this period and that for 90% of the period the rainfall was 10 points or less (100 points = 1 in.). The highest rainfall shown in the figure is 250 points, although on one day 349 points of rain was actually recorded. In terms of rainfall, therefore, the weather conditions seem favourable for drying farm products. To predict whether certain periods would be suitable for the drying of groundnuts in particular, information on atmospheric humidity would also be necessary.

Humidity.—Fig. 4 shows the R.H. likely to be encountered in Kingaroy during the individual months of the harvesting season. The ordinate represents that percentage of time during which the atmosphere did not exceed a particular relative humidity indicated by the abscissa. The Figure shows that the humidity during the harvesting season could range between 20 and 100%.

Interpretation of the sorption isotherms

Fig. 5 shows sorption isotherms between 54 and 99% R.H. The isotherm at 25°C lies below the one at 15°C. This tendency has also been reported by others[15–17] and, since sorption processes are generally exothermic, is in agreement with Le Chatelier's principle.

These sorption isotherms indicate that an atmosphere of 15–25°C (59–74°F) should have a humidity of less than 75% to dry groundnuts to a safe storage moisture of about 10–12%.

According to the sorption isotherms measured for the shells and kernels separately (Fig. 6), equilibrium moisture contents were higher for shells than for kernels in the 54–92·5% R.H. range. Between 92·5–99% R.H., equilibrium moistures were higher in the kernels. Karon & Hillery[18] measured sorption isotherms for Virginia Runner groundnuts and observed higher equilibrium moistures for shells than for kernels at humidities from 11·1 up to 92·5%. At 92·5% R.H. they found that shells and kernels had the same equilibrium moisture content of about 25%, in good agreement with the 23% resulting from the present investigations (Fig. 6).

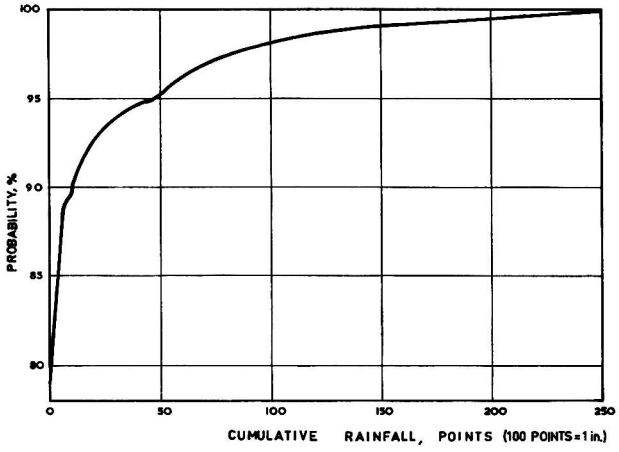

FIG. 3.—*Probable rainfall in Kingaroy, April, May and June collectively*

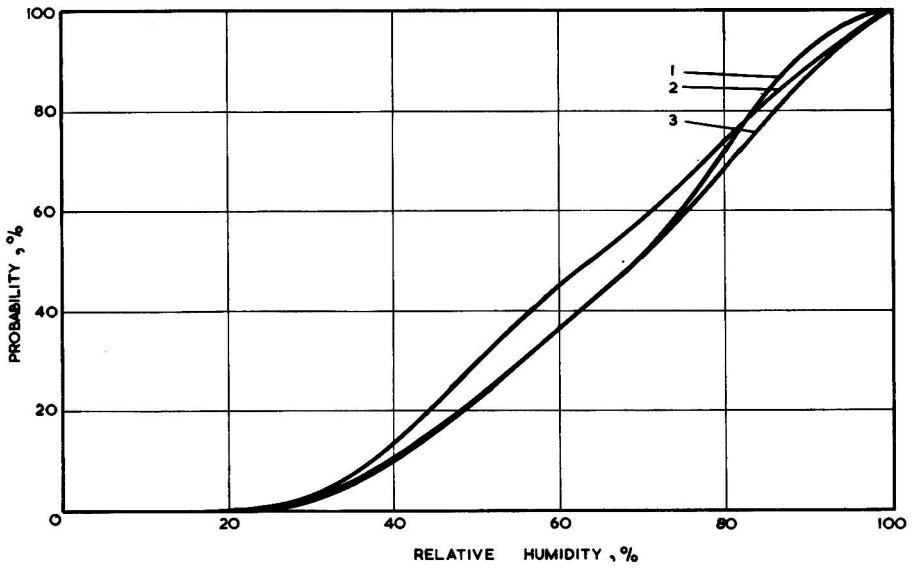

FIG. 4.—*Probable humidity in Kingaroy, April* (curve 1), *May* (curve 2), *and June* (curve 3), *individually*

The difference in sorptive capacity between groundnut shells and kernels is no doubt caused partly by the closer pore structure of the kernels and partly by its different chemical composition. A high hemicellulose content of the shell[19] is one reason why it is more hydrophilic[20] than the kernel and has a larger sorptive capacity in the lower humidity range. At higher R.H. the sorptive capacity of most materials is more dependent on pore structure since condensation can occur more readily in the finer capillaries. This would account for the greater relative sorption of moisture by the kernels at higher relative humidities, as compared with the coarser-pored shells. Owing to their high oil content (45–50%),[21] groundnut kernels, like other oilseeds, have much lower equilibrium moisture contents than have cereals, e.g. wheat[22] and rice,[23, 24] over a large range of R.H.

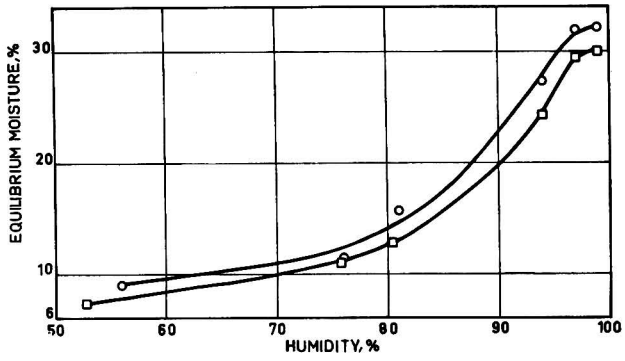

FIG. 5.—*Sorption isotherms for the whole groundnut*

○ 15° C □ 25° C

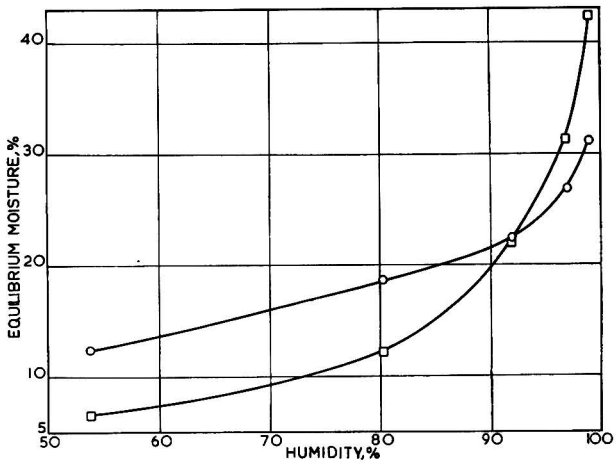

FIG. 6.—*Sorption isotherms for shell* (○) *and kernel* (□) *of groundnut*

Air drying studies

Humidity probability curves of the atmosphere and sorption isotherms of a material enable one to predict whether that material could be dried successfully in the atmospheric conditions prevailing. Data on which Figs. 4 and 5 were based were combined to produce Fig. 7, in which the ordinate indicates the probability that groundnuts may be dried during the season to at least the moisture on the abscissa. It shows that for about 63% of the total time available the air humidity would be suitable for drying groundnuts to a safe storage moisture of 21%. It follows that if artificial drying with unheated air were to be used, the air would be too moist for 37% of the time and could not be utilised during the last stages of drying. Not only would it fail to remove moisture from the nuts, it would actually add water to them if it were admitted when the crop was almost dry.

Pilot plant operation.—In one of the most instructive drying experiments, the fan was operated to deliver 850 cu. ft. of air/min. (measured at 60°F and 760 mm. Hg.), or about 21 cu. ft. air/(min.) (cu. ft. load). This was based on a number of preliminary trials with various dryer designs, a description of which is beyond the scope of this article. The air was admitted to the dryer at a temperature of about 100°F and an average relative humidity of about 14%.

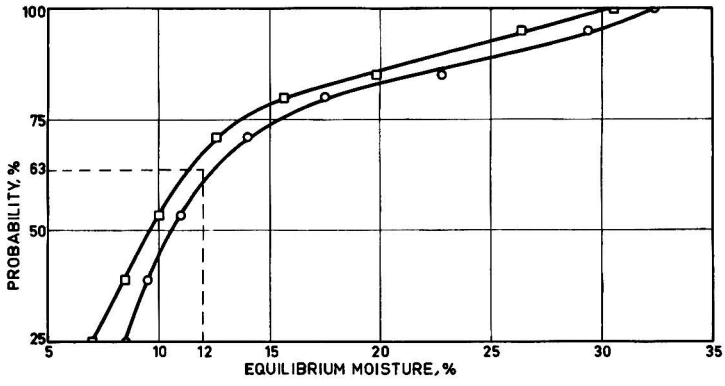

FIG. 7.—*The extent of drying groundnuts with unheated air*
○ 15° C □ 25° C

Table I shows how temperatures along the axis of the load were distributed during the experiment. In the beginning there were large temperature differences between lower and upper levels, while at the end the difference was only 4°F. These conditions would produce moisture gradients in the crop during the early stages of drying, whereas the moisture content of the finished product would tend to be more uniform.

Moisture variations in the load during one experiment are demonstrated by Fig. 8. Test position 4 was 1 in. below nut level at the centre, and positions 5 and 6 were 3 ft. 2 in. and 10 in., respectively, below nut level at the dryer side. A maximum moisture difference of about 10% was observed between upper and lower test positions after about 10 h. of drying; then moisture gradients decreased and after about 29 h. a moisture difference of about 2% was observed. A similar equilibration of moisture during drying of other crops has been reported by Hughes & Mitchell.[25]

The results of the pilot studies showed that:

(1) Artificial bulk drying of groundnuts, carried out with air heated to 100°F, resulted in a product which, in appearance and flavour, could compete with groundnuts dried under the best conditions in the field.
(2) Drying the crop from a moisture content of 31% to one of 12% could be accomplished in 24 h. by a specific air flow of about 21 cu. ft. air/(min.) (cu. ft. load).

From a physical point of view, the air temperature influences the speed of drying by adjusting the driving potentials (temperature and vapour pressure gradients) for heat-and mass-transfer. The air flow also has a dual effect on the rate of drying. On the one hand, it influences

Table I

Temperature measurements on pilot-scale dryer

Time, h.	Position*:	Air temperature, °F					
		1	2	3	4	C	G
0		55·4	56·1	58·4	56·1	71·8	
1		90·4	75·4	60·6	59·5	99·0	66·5
3		94·0	87·1	75·6	74·2	99·6	58·0
6¼		91·0	84·7	78·0	77·3	96·8	43·0
17		95·8	94·6	92·7	92·1	97·4	38·0
20		99·5	99·0	98·9	99·5	101·4	59·0
23		98·9	99·1	97·6	96·3	100·5	66·0
26		98·4	98·2	96·4	94·7	100·1	65·0
29		96·0	94·4	93·0	92·0	97·8	50·0

*Symbols are as in Fig. 1. (C shows the average of the temperatures in four ducts.)

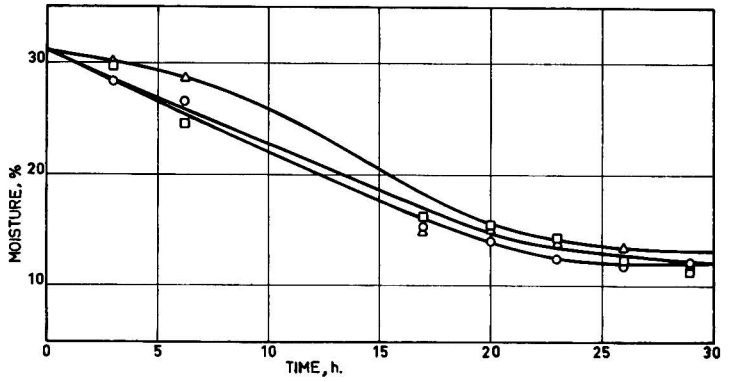

FIG. 8.—*Moisture variations of the load during the experiment*
○ position 5 □ position 6 △ position 4

the concentration of water vapour in air interstices of the load, and thus modifies the driving potential for moisture transfer. On the other, it affects the coefficients of heat- and mass-transfer, which for some materials are proportional to the air velocity raised to the 0·8 power.[26, 27] Because of this interrelationship between temperature and flow of the air, one factor may be decreased at the expense of the other. The controlling factor will be the temperature, and stringent evaluation of the product quality is recommended if air temperatures in excess of 100°F be used.

Performance of the larger prototype dryer.—Fig. 9 shows how temperatures were distributed along the vertical axis of the load during drying. Thermocouples Nos. 19, 20, 21 and 22 were located 6 in., 3 ft., 5 ft., and 7 ft. respectively above floor B. The temperature in the load 6 in above the floor was usually 25–30°F higher than that of the atmosphere, which ranged between 64 and 81°F. The average R.H. of the air at this point was about 20%. Large temperature gradients in the axial region of the load were evident during the first half of the drying period. After about 20 h. the temperature difference was reduced to 3°F or less which, according to the pilot run, indicated that the end of drying was approaching.

Measurements of temperatures in the load near the corners showed small variations during the test. One corner was consistently warmer (by as much as 3°F) than the others. This was most likely due to the fact that it was situated on the leeward side and had a lower rate of heat loss.

Moisture variations in four locations of the crop are presented in Fig. 10, where sampling points M_1, M_2, M_3 and M_4 were 6 in., 3 ft., 5 ft., and 7 ft., respectively, above the perforated floor. Moisture differences in the crop, already present at the beginning of the test, increased slightly during the first 15 h. of drying, then decreased and gradually approached about 3% at the end of 24 h.

The curves in Fig. 10 demonstrate two important principles of contemporary drying theory:

(1) when the moisture of a material is below a critical value, the rate of drying decreases with decreasing moisture.
(2) the driving force associated with drying is the difference between the humidity at the material surface and the humidity in the surrounding air.

A comparison of initial conditions in various locations of the load shows that M_1 had the lowest moisture content (26%) and, being 6 in. above the floor, was in contact with the driest air. These two conditions, according to principles (1) and (2) above, have opposite effects on the relative rate of drying at M_1. During the first 15 h., for example, the rate of drying was only slightly

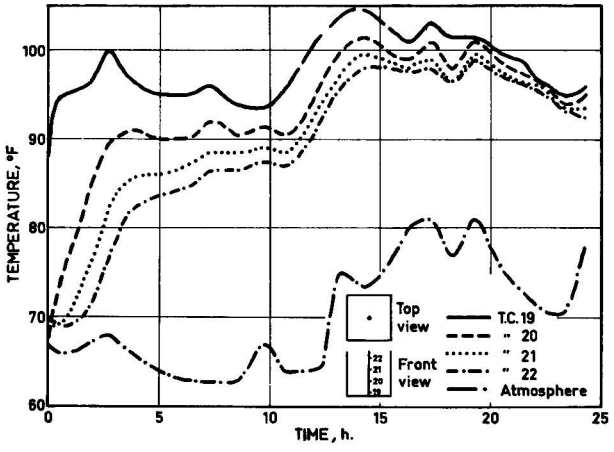

FIG. 9.—*Temperature variation along dryer axis*

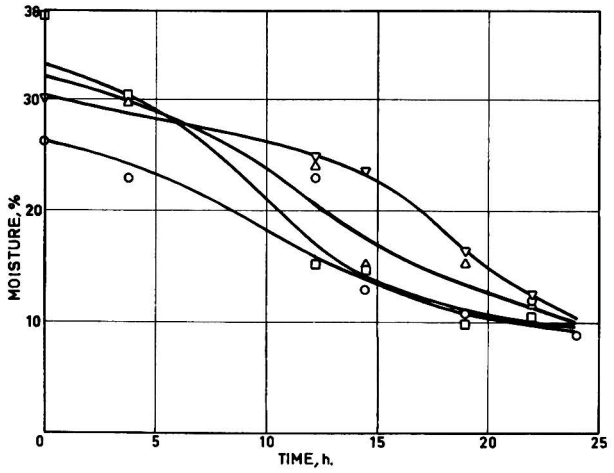

FIG. 10.—*Moisture variation in load*
Location ○ M1 □ M2 △ M3 ▽ M4

higher at M_1 than in the top location M_4. The nuts at M_4 started at a moisture content of 30% and were in contact with the wettest air, conditions which, in comparison with those at M_1, also have an opposite influence on the rate of drying.

Nuts in locations M_2 and M_3 started with moisture contents of 33 and 32%, respectively, and drying air at M_2 had the lowest humidity. As would be expected from the principles cited, the initial rate of drying was higher at M_2 than at M_3. However, after 15 h. the moisture content of the load had decreased so much at M_2 that the rate of drying there stayed lower than at M_3 for the rest of the test. During the last 9 h. of the test a gradual decrease of moisture in the lower levels of the load (M_1–M_3) resulted in gentle drying, i.e., slow transfer of moisture to the flowing air. For this reason the high-moisture load at the top (M_4), which was in contact with air of an increasing drying potential, attained the highest rate of drying during this period, at the end of which moistures of groundnuts at the four sampling points ranged between 9 and 12%.

Fuel consumption for the diesel-driven heater and fan utilised in this dryer was 2·2 gal./h. at a fuel cost of 24d.(Australian)/gal. Thus a load of about 7 tons (wet) could be dried in 24 h. at a fuel cost of about 0·12d.(Australian)/lb. of product.

Conclusions

The foregoing results indicate that in the climate prevailing at Kingaroy during the harvesting season a combination of natural curing and artificial drying provides a feasible means of economically reducing the moisture content of groundnuts to a safe storage value. Curing in windrows reduces the moisture content to about 25–30%, for which the atmospheric conditions are suitable for about 90% of the drying season (Fig. 7). The high water content of the crop during curing results in a reasonably high rate of drying, and evaporative cooling is adequate to prevent overheating of the crop on specially hot days. At the end of the natural curing period, when the rate of drying has decreased considerably, artificial drying under controlled conditions to a final moisture content of 9–12% is necessary.[8] The slow exchange of heat and moisture between the well-insulated groundnut kernel and its surroundings is one reason why artificial drying should be carried out with heated air. Beasley[28] has suggested the compromise of applying heat only when the ambient atmosphere exceeds a humidity of 65% which, in the main groundnut growing area of Australia, would amount to about 50% of the total drying time. This might be acceptable to growers who were prepared to meet the cost of control instruments and who were not in a hurry for the dried product. However, uninterrupted application of heat

during the whole period of artificial drying has been recommended by others.[8, 9, 10, 29] In the equipment described above, air heated to about 100°F and flowing at about 20 cu. ft. air/(min.) (cu. ft. load) would dry the groundnuts from a moisture content of 30% to about 12% in 24 h.

Proper planning on the larger mechanised farms (about 100 acres or more of groundnuts) would require that an artificial dryer be operated to keep pace with threshers, which can normally handle 500–600 bags of nuts per day. A dryer with this capacity would need a volume of about 2,000 cu. ft. and should be operated at the highest temperature compatible with a good product.

The trend towards bulk handling of groundnuts suggests that artificial drying in bulk may prove economically attractive. This is also more efficient than drying in bags.

Acknowledgments

The author gratefully acknowledges the help and cooperation from Messrs. O. Higgins, D. Marshall and A. Baikaloff, Peanut Marketing Board, Kingaroy, Queensland, and Messrs. M. & R. Champney, Wooroolin, Queensland; the assistance rendered by Misses J. Hayhurst and N. Taylor, Commonwealth Scientific and Industrial Research Organization; and the financial support received from the Peanut Marketing Board.

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Received 22 February, 1965, amended manuscript 21 July, 1965

References

[1] Morris, N. J., & Dollear, F. G., 'Abstract Bibliography of the Chemistry and Technology of Peanuts, 1830–1939', *U.S. Dep. Agric., Bur., agric. industr. Chem.*, 1949, AIC–151
[2] Jones, M. A., 'Peanut Research at the Southern Utilization Research and Development Division, New Orleans, Louisiana, 1942–1961', 1962 (U.S. Dep. Agric: agric, Res. Service)
[3] Barringer, K. L., *J.N.Y. bot. Gdn*, 1955, 5(1), 6
[4] *Production Yearbook*, 1963, **17**, 120 (Rome: F.A.O.)
[5] Sreenivasamurthy, V., & Jayaraman, A., *Fd. Sci., Mysore*, 1963, **12**, 290
[6] Diener, U. L., Davis, N. D., Salmon, W. D., & Prickett, C. O., *Science*, 1963, **142**, 1491
[7] Blatchford, S. M., & Hall, D. W., *Trop. Sci.*, 1963, **5**, (2), 82
[8] Teter, N. C., *Agric. Engng, St. Joseph, Mich.*, 1954, **35**, 568
[9] Sorenson, J. W., *Agric. Engng, St. Joseph, Mich.*, 1948, **29**, 397
[10] Baker, V. H., Cannon, B. M., & Stanley, J. M., *Agric. Engng., St. Joseph, Mich.*, 1952, **33**, 351
[11] Pickett, T. A., & Holley, K. T., 'Chemical Studies of Peanut Curing', *Mimeo Ser., Georgia agric. Exp. Sta.*, 1960, N.S. No. 90
[12] Robinson, R. A., & Stokes, R. H., 'Electrolytic Solutions', 1955, (London: Butterworths)
[13] Weyler, A., & Hasegawa, S., *J. Res. nat. Bur. Stand., Wash.*, 1954, **53**, 19
[14] Hoffpauir, C. L., *Oil and Soap*, 1945, **22**, 283
[15] Glasstone, S., 'Textbook of Physical Chemistry', 2nd edn., 1947, p. 1196 (New York: D. Van Nostrand Company Inc.)
[16] Kelsey, K. E., *Aust. J. appl. Sci.*, 1957, **8**, 42
[17] Ross, S., & Clarke, H., *J. Amer. chem. Soc.*, 1954, **76**, 4291, 4297
[18] Karon, M. L., & Hillery, B. E., *J. Amer. Oil Chem. Soc.*, 1949, **26**, 16
[19] Radhakrishnamurthy, B., & Srinivasan, V. R., *J. sci. industr. Res.*, 1961, **19C**, 157
[20] Sadoh, T., & Christensen, G. N., *Aust. J. appl. Sci.*, 1964, **15**, 297
[21] Robbins, W. W., 'The Botany of Crop Plants', 3rd edn, 1931, p. 444 (Philadelphia: P. Blakiston's Son & Co., Inc.)
[22] Gay, F. J., *J. Coun. sci. industr. Res. Aust.*, 1946, **19**, 187
[23] Houston, D. F., *Cereal Chem.*, 1952, **29**, 71
[24] Juliano, B. O., *Cereal Chem.*, 1964, **41**, 191
[25] Hughes, J. M., & Mitchell, T. J., *J. Sci. Fd Agric.*, 1959, **10**, 39
[26] Shepherd, C. B., Hadlock, C., & Brewer, R. C., *Industr. Engng Chem.*, 1938, **30**, 388
[27] Myklestad, O., *Chem. Engng Progr. Symp. Ser.*, 1963, **59**, (41), 129
[28] Beasley, E. O., Proc. 1st Int. Symp. Humidity Stat litr. and Moisture, vol. 2, 1965, p. 126 (New York: Reinhold Publishing Corp.)
[29] Gronert, A., U.S. P. 2,245,664

A SIMPLE METHOD FOR DETERMINING SMALL AMOUNTS OF FORMALDEHYDE IN SILAGE*

By H. NEUMARK

A method is described by which formaldehyde, present in silage, is precipitated by 2,4-dinitrophenylhydrazine and the formaldehyde, liberated from the hydrazone by hot concentrated sulphuric acid, is assayed photometrically with chromotropic acid reagent.

Introduction

In the course of studies carried out in this laboratory on the fermentation and palatability of silage for cattle, it was found that the presence of formaldehyde was connected with undesirable fermentation processes, and that the compound itself greatly influences the appetite of ruminants.[1] Because of the low concentration of this aldehyde in silage, however, considerable difficulties were always encountered in carrying out precise quantitative determinations.

The quantitative determination of formaldehyde as 2, 4-dinitrophenylhydrazone by paper chromatography requires the separation of the free 2, 4-dinitrophenylhydrazine from the hydrazones. According to the method of El Hawary & Thompson,[2] this is done by centrifugation, but as small amounts of hydrazone remain in the solution, this method is unsuitable when the aldehyde is present in only very low concentrations.

The assay of formaldehyde by gas chromatography becomes very difficult because of the ready polymerisation of this compound at higher temperatures.

Direct colorimetric determination of the aldehyde by the method of West & Sen[3] in deproteinised extracts is not suitable, as the fluid contains materials which undergo reaction with the reagent.

In order to carry out the chromotropic acid test, the formaldehyde must be separated by distillation, a procedure involving losses, the extent of which is dependent upon the conditions under which formaldehyde is distilled.[4] The detection of small amounts of formaldehyde by the method of West & Sen[3] is further limited by the fact that a concentration of at least 86% sulphuric acid is required for optimal colour development, which means that 3 c.c. of the whole distillate is the maximum aliquot with which the reaction can be carried out.

In order to overcome the above-mentioned difficulties and to provide a reliable method for detection of very small amounts of formaldehyde in silage, a procedure was developed which is based on the reaction between hot concentrated sulphuric acid and the 2, 4-dinitrophenylhydrazone of formaldehyde.[5]

Experimental

The ensiled material (sorghum, sugar beet tops, molasses + clover, green peas with orange peels) (about 30–40 g. of fresh silage) was homogenised in a Waring Blendor, with 200 ml. of sodium tungstate solution (20 ml. of 10% sodium tungstate + 20 ml. of 0·6 N-sulphuric acid + 60 ml. of water).

The slurry was filtered through cheese cloth, the deproteinised filtrate centrifuged and the supernatant decanted. About 20 ml. of the solution were decolorised with a small amount of active charcoal (Darco Practical, Eastman Kodak Co.) by shaking in a 50 ml. Erlenmeyer flask several times for short periods. [It was established that the charcoal did not absorb any formaldehyde from the solution (see Table I).]

After about 5 min. the contents of the flask were filtered through a paper filter and 1 ml. of a solution of 0·2% dinitrophenylhydrazine in 2N-hydrochloric acid was added to 10 ml. of the filtrate; the whole solution was then incubated for 20 min. at 38°. The solution was then extracted three times with 50 ml. ethyl acetate and the combined extractions were centrifuged

* Contribution from The National and University Institute of Agriculture, Rehovot, Israel. 1965 Series, No. 859-E.

Table I

Optical density of the chromotropic acid reaction product obtained from formaldehyde solutions before and after treatment with Darco carbon

Formaldehyde, μg.	Optical density at 570 mμ	
	Untreated	After carbon treatment
5	0·115	0·115
10	0·238	0·241
20	0·486	0·473

to remove any emulsion. The clear ethyl acetate solution was washed twice with 30 ml. portions of 10% sodium carbonate to remove material interfering with the chromotropic acid reagent. The ethyl acetate solution was again centrifuged to remove all traces of water, and the organic phase was decanted and evaporated to dryness at 60° *in vacuo*. The yellow precipitate was dissolved in 2 ml. of water, then 2 ml. of the chromotropic acid reagent were added, followed immediately by 14 ml. of conc. sulphuric acid. After the colour had developed, the acidic solution was transferred to a 25 ml. volumetric flask and made to volume with water. The optical density of the colour was read at 570 mμ in a Beckman spectrophotometer against a blank containing all the reagents. In assaying legume silages, a slight turbidity appears at the final solution, which is readily removed by centrifugation at 2500 r.p.m.

Results

A calibration curve for optical density at 570 mμ with known amounts of formaldehyde (0–20 μg.) gave a straight line through the origin, indicating that Beer's Law is followed.

Table II shows that the recovery of the added formaldehyde was very good, and that silage additives such as molasses did not interfere in the procedure, but were successfully removed by the purification steps described.

Table II

Recovery of formaldehyde added to silage extracts

Silage	Formaldehyde p.p.m. in fresh material			Recovery, %
	Present in silage	Added to silage extract	Total found	
Sorghum	4·7	0·7	5·4	100
Clover + 3% molasses	0·8	19·5	18·8	92·6
Peas and orange peels	0	19·5	19·2	98·4

Discussion

In recent years the micro-assay for formaldehyde has grown in importance, as it plays an important role in biological processes, e.g., in synthesis of purines and formation of formyl-tetrahydrofolic acid. The toxicity of formaldehyde prevents a greater concentration of this compound in biological systems—and the difficulties in assaying micro-quantities of this compound may be overcome by the above described method. By the procedure described it is possible to assay 0·04 p.p.m. of formaldehyde and even less, as the upper limit of fluid which can be assayed may be about 40 ml.

Acknowledgment

The author wishes to thank Prof. A. Bondi of the Faculty of Agriculture, Rehovot, for his interest in and helpful suggestions regarding this work.

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Received 25 March 1965

References

[1] Neumark, H., Bondi, A., & Volcani, R., *J. Sci. Fd Agric.*, 1964, **15**, 487
[2] El Hawary, M. F. S., & Thompson, R. H. S., *Biochem. J.*, 1953, **53**, 340
[3] West, P. W., & Sen, B., *Z. anal. Chem.*, 1956, **153**, 177
[4] Walker, J. F., in 'Formaldehyde', 3rd edn, 1964, pp. 503 (New York: Reinhold Publishing Corp.)
[5] Sawicki, E., Hauser, T. R., & McPherson, G. N., *Analyt. Chem.*, 1962, **34**, 1460

A RECORDING INSTRUMENT FOR THE RAPID EVALUATION OF THE COMPRESSIBILITY OF BAKERY GOODS*

By A. T. S. BABB

A recording dynamometer has been developed for the rapid examination of samples for firmness and compressibility. The instrument has two essential parts: (1) a platform which forces the sample against a compression block and (2) a measuring section using a strain gauge, which converts the forces as they develop into recordable electrical signals. Interchangeable compression blocks allow for wide variations of texture. Up to 27 samples of uniform thickness may be tested in 1 min. and the slices comprising 40 large loaves of bread may be examined within an hour. The peak forces developed are recorded and can readily be converted to modulus of axial compression. There are attachments for toughness measurements and for snap tests on products such as biscuits.

Introduction

With progress in technological control in the food industry, the need for objective assessment of the physical properties associated with staling, variously described as compressibility, texture, toughness, shortness etc., has increased. In the baking industry the need was found for a rapid method of testing such that a large number of results, suitable for statistical treatment, could be obtained in a period of time during which staling was negligible. Such a method would be convenient for determining variations throughout a batch or within an individual sample.

In general, compressibility is determined either by measuring the penetration of a suitable probe under constant load (softness value) or by determining the force required to cause a definite deformation (firmness value). In studying bread staling the softness value has been adopted by several authors.[1–3] The firmness value on the other hand has been used by other authors[4,5] in studying staling changes of bread and of cake. Brice & Geddes[4] compared the two techniques and preferred the firmness method by which the results can be expressed as modulus of axial compression measurements, which are related to those of Young's modulus of compression.

In the instrument to be described, which uses the firmness technique, one can carry out 27 compression tests per minute or alternatively, using a special attachment, shear and toughness measurements (cutting out a disc of the material) may be made followed by the above mentioned compression tests. In the latter case 10 combined measurements may be made in a minute.

* Read at meeting of Food Group, 11 November, 1964

Experimental

A. *Description of the instrument*

The tester consists essentially of a powered compression device and a measuring section utilising a strain gauge for converting the forces developed, when the sample is being compressed or sheared, into measurable electrical signals. These signals, after passing through a control box, are fed into a high speed recorder (see Fig. 1).

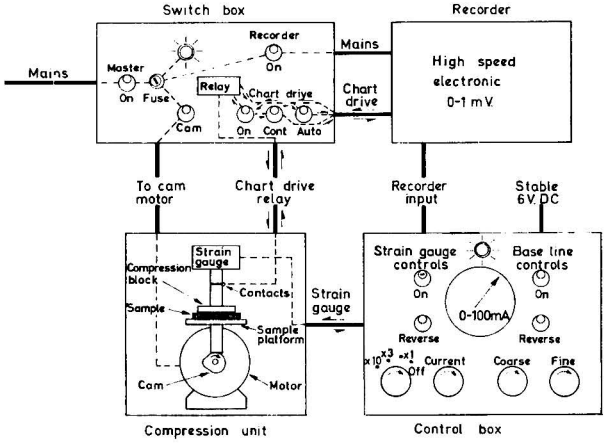

FIG. 1.—*Schematic diagram of apparatus*

(1) *Compression device.*—This section, which is illustrated in Fig. 2, contains the sample platform which is raised against a compression block by an interchangeable linear velocity cam whose speed can be varied between 3 and 27 r.p.m. The cam in use is mounted on the output shaft of a gearbox driven by and carrying a powered Kopp Variator (size 12 type MSRI, All-speeds Ltd., Acton), a device for giving a stepless control of speed. Above the platform is situated an interchangeable compression block which transmits to the strain gauge, via its mounting rod, the forces developed during the tests. The strain gauge and its safety weight above it, are carried on a balanced and hinged platform which rises in the event of an excessive force being applied, and calibration is effected by the addition of the appropriate weights after first removing the safety weight.

To make the instrument suitable for examining samples of widely different compression and shear properties the following provisions were made in the design: (1) the gap between the sample platform and the compression block can be adjusted by changing the position of the latter on its mounting rod; (2) the amount by which the sample is raised can be altered by changing the cam; (3) compression blocks of 10, 20, 40 and 80 mm. dia. are used. All parts except the cam are supported by a vertical section of 3 in. iron channel, which is itself rigidly mounted on similar but horizontally disposed channel pieces to form a base plate, which also carries the gearbox.

The cams used for raising the sample platform need special mention. They are of the linear velocity type and are so shaped (see Fig. 3) to give a low stationary phase of 60° for introducing the sample, a linear rise phase of 220° to give the compression and a high stationary phase of 60° to allow, if necessary, time for the recorder to catch up.

The recorder chart movement is controlled by low-voltage contacts, which are actuated by the compression block and which close immediately compression of the sample commences. These contacts energise a relay and allow, with suitable panel switches, the following choice of chart movements: (1) continuous, (2) during compression only, (3) between compression only and (4) no movement. Fig. 4 shows movements (1), (2) and (3)..

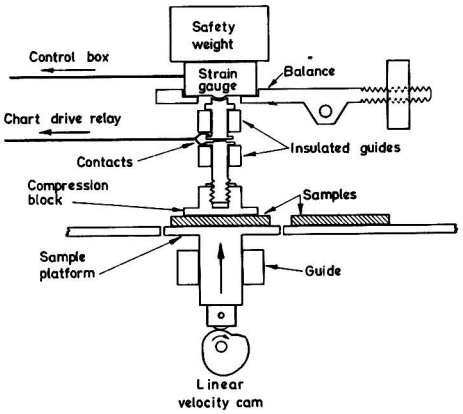

FIG. 2.—*Compression unit*

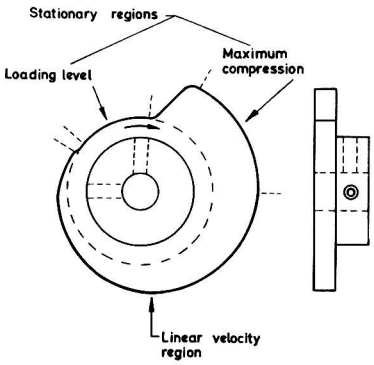

FIG. 3.—*Linear velocity lifting cam*

The complete lifting mechanism is fixed to the top of a trolley and is enclosed in a detachable box (see Fig. 5), which has a flat top through which the sample platform is raised during the test, and along which the sample is slid into position for the test. A control knob is located on the front of the box for adjusting the cam speed, and consequently, the testing rate.

For the simultaneous measurement of the compression and breaking tangential (shear) forces, use is made of special Perspex frames, each of which takes up to 6 test slices (see Fig. 6). Each frame is made up of two long strips with six pairs of aligned holes placed in a row and slightly larger than the compression block used. The thickness of the lower strip, the one on which the test slices are placed, is slightly greater than that of the slices, and that of the upper strip, which acts as a guide for positioning under the compression block, is $\frac{1}{16}$ in.

(2) *Measuring section.*—The forces developed, when the sample is being tested, are obtained with the aid of a strain gauge which gives an output voltage proportional to the force applied to it. The strain gauge used, a Saunders Roe type B range 0–50 lb., is energised with a stable d.c. voltage via a control box, which also accepts its output and attenuates it to a value suitable for passing on to the recorder.

The control box (see Fig. 7) has three main functions: (1) it provides, by means of the fine and coarse controls R_9 and R_{11}, an adjustable shift voltage for setting the recorder base line; (2) with the aid of the control R_1 it decides the value of the strain gauge energising current;

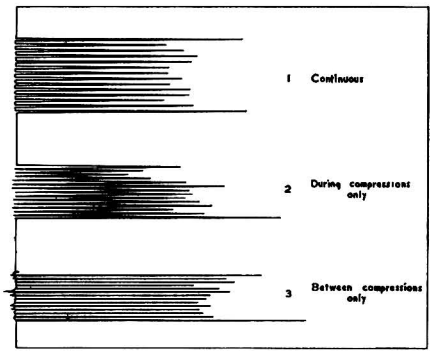

FIG. 4.—*Types of chart movement* (1) *continuous,* (2) *during compressions only* (3) *between compressions only*

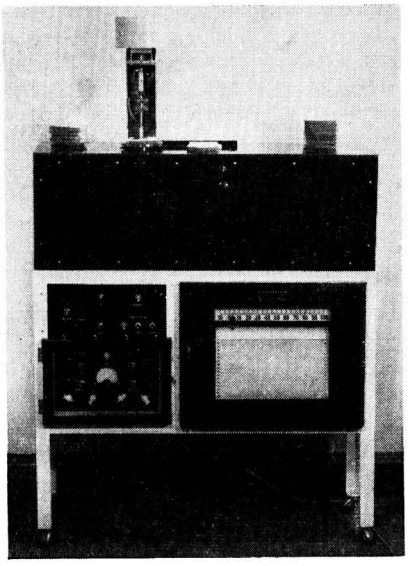

FIG. 5.—*The complete apparatus*

(3) by means of the preset control R_2 and the attenuator, it selects a portion of the output voltage suited to the sensitivity of the recorder.

The control R_2 is a standardising pre-set resistance which has been so adjusted that, with no signal attenuation and an energising current of 80 mA, a change in the applied force of 1 lb. will cause a change in the recorder reading of 10 divisions. Under similar conditions but with an energising current of 40 mA, a force of 2 lb. will be necessary to give the same deflection; the sensitivity has thus been halved.

The signal attenuator comprises the rotary switch S_3 and the potentiometer resistance chain R_3 to R_7; two of these resistances, the preset ones R_4 and R_6 have been so adjusted that the signal can be reduced to $\frac{1}{3}$ or $\frac{1}{10}$, giving correspondingly reduced deflections and sensitivity. The recorder is scaled -10 to $+100$ divisions and the recorded readings can be converted to forces expressed as pounds by means of a simple conversion factor.

The purpose of the high-value shunt resistance R_{12}, connected across one of the Wheatstone bridge elements of the strain gauge, is to correct for slight asymmetry, thus ensuring that the signal output from the gauge is zero when no force is applied to it. The recorder base line is then not affected when the attenuator and strain gauge current settings are changed.

The recorder used, a Honeywell–Brown Electronic, has a sensitivity of 1 mV and a response

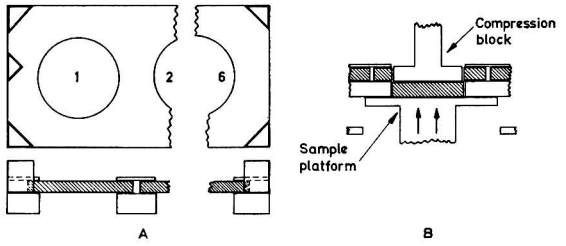

FIG. 6.—*Combined shear and compression test*

A holder B holder in use

Note. The test slices are shown hatched. In B the compression test is being made immediately after the shear test

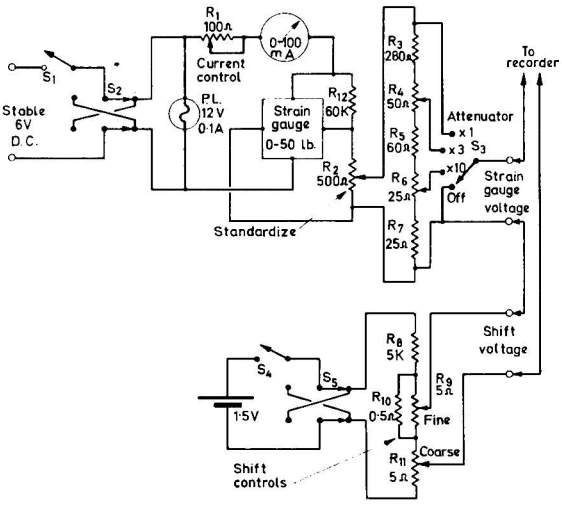

FIG. 7.—*Control box circuit*

time of 1¼ sec. both for full scale deflection; eight chart speeds in the range 10 in. to 1440 in. per h. can be selected.

Two stable d.c. voltages are required, one from a 6-V 10 amp.-h. accumulator for supplying the energising current to the strain gauge, and the other of 1·5 V from a U.2 dry battery for the zero shift voltage.

The control box and the recorder are housed in the trolley below the compression section and can be readily removed. Above the control box is a panel supporting the fuses, the master switch, and switches for the cam motor, for the recorder and for the recorder chart movements.

(B) *Method of operation*

Preliminary adjustments.—Before tests can be made it is necessary to decide on the depth of penetration and on the area of the compression block. These factors will obviously depend on the nature of the slice to be tested, thick ones needing a cam giving a greater lift to the sample platform, while with soft slices an increased sensitivity may be needed and can be obtained by using a large diameter compression block. When testing extra firm slices and also some thick ones, it may be necessary to use the small-diameter compression block to prevent too great a force being applied to the strain gauge. After fitting of the appropriate cam and compression block, the height of the latter is adjusted to give the required penetration. With a new product, a few preliminary runs should be made to decide on the attenuator setting, the strain gauge current, the testing rate and the recorder chart speed.

Although with a little practice it is possible to feed by hand up to 27 slices per minute to the tester employing a standardised drill, the testing rate decided on was 24 slices per minute. The rate is limited by the speed of response of the recorder pen and this makes it necessary, when the recorder deflection is more than 50% of full scale, to reduce the travel speed of the pen by halving the energising current to the strain gauge or by attenuating the signal. A similar effect could also be obtained, without reducing the deflection, by halving the testing rate and then halving the chart speed to give the same dimensional display.

It was found in practice that a cam giving a rise of 15 mm. and a 40 mm. dia. compression block, adjusted to give a penetration of 5 m. to a 10-mm. thick slice, were suitable for most products.

Firmness test.—The following procedure was found to be satisfactory for the rapid testing of all the slices of many sliced loaves of bread.

After applying the mains to the tester, the recorder is switched on and the energising voltage to the strain gauge and the shift voltage applied. After allowing 5 min. for the circuits to stabilise, the strain gauge current is adjusted, the attenuator setting is selected and the shift voltage adjusted to set the recorder base line. The chart speed is selected and then the chart drive switches set so that the chart moves continuously except when compression is actually occurring, checking by applying a small upward force to the compression block and noting that the chart ceases to move. The Variator driving the cam is now switched on and its speed adjusted till the cam rotates at 24 r.p.m. The instrument is now ready for making the tests.

Each loaf is now tested employing the following procedure (see Fig. 5). After removal from its wrapper, the loaf is placed vertically on the flat top of the tester with its base towards the back and the top slice (crust), is discarded. The remaining slices are tested consecutively until the other crust is reached, this also being discarded. Each slice is placed in turn on the flat top with its base crust against the guide rail and pushed on to the sample platform, during the stationary period at the end of its downward travel, while at the same time pushing out the previous slice. As the platform rises, the sample engages the compression block and immediately compression commences the relay is energised causing the chart to cease moving. The platform continues to rise and the force developed during the compression is recorded. After the platform falls, de-energising the relay and thereby switching on the chart motor, the next slice is similarly moved into the testing position and the process continued until all the slices of the loaf are tested. Then the whole procedure repeated until all the remaining loaves are tested.

The method is very suitable for the rapid testing of all the slices from a large number of loaves. For example, 40 large loaves may be examined in 1 h. to give a concise record with the results for the slices of each loaf displayed in individual groups. This is illustrated in Fig. 8 in which numbers 17 to 31 each correspond to the slices of one loaf.

Combined shear and firmness tests.—Slices of uniform thickness are loaded in the holders as previously described (see Fig. 6). After each test the holder is moved across the platform to the location of the next slice while the platform is in the lower stationary phase, being careful to ensure that each alignment hole is accurately positioned under the compression block. It is necessary during these tests to make the chart move during the actual shear and compression operation to ensure that the recorded peak forces are separated on the chart, which remains stationary between the individual sample tests. In this combined test the force required to shear off the disc is first recorded and this is immediately followed by a firmness test on the resulting disc (see Fig. 9).

(C) *Method of presentation of results*

For most purposes the results obtained with this apparatus need only be comparative, but if desired the figures for ' firmness ' may be converted to the Modulus of Axial Compression, as the thickness, depth of penetration, and the compression area of the slice of the material are all known (see Appendix). As will be demonstrated, the ' firmness ' figures on slices taken near the outside of the bread are usually somewhat higher than those from the centre. It has been our practice therefore to limit determinations to centre slices, the results of which are averaged.

Applications

(1) *Bread: firmness measurements*

(a) Fig. 10 shows some typical compression force traces for fresh and stored bread. They indicate the variations in firmness from end to end of the loaves and also the increase in firmness with time of storage.

(b) Fig. 11 shows the increase in firmness as expressed as the value for the modulus of axial compression of four different types of bread with storage at room temperature up to 6 days.

(2) *Cake: shear and firmness measurements*

(a) Fig. 12 shows the shear and compression force traces for fresh and stored madeira cake. The traces again indicate the variations in firmness across the cake and also the increase of both forces with storage.

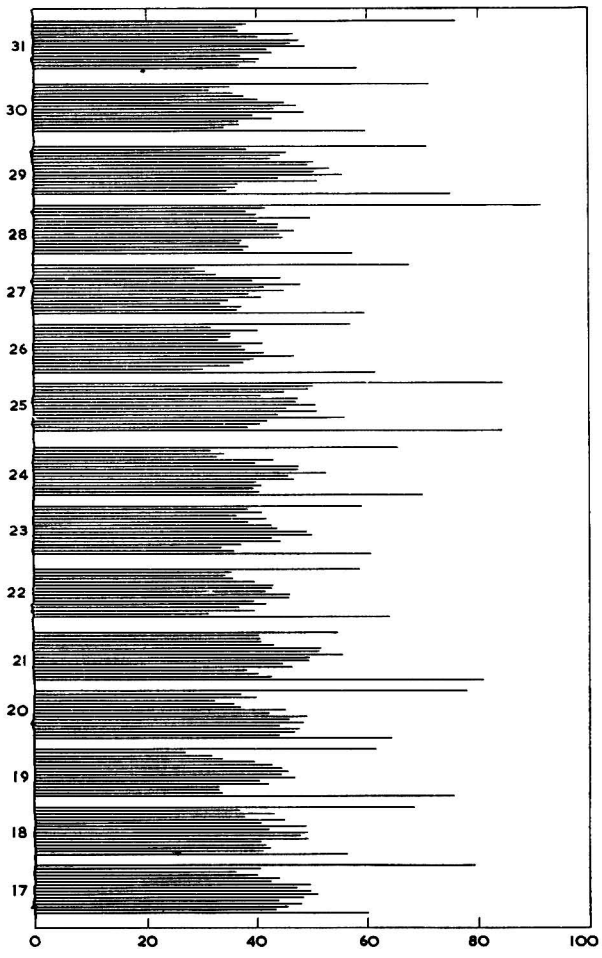

FIG. 8.—*Recorder chart of rapid compressibility tests on bread*
(All slices of each loaf except the crust were tested consecutively)

(b) Fig. 13 shows that, as with bread, an increase occurs in the value of the modulus of axial compression for Madeira and sponge cakes when stored at room temperature.

(3) *Biscuits: 'snap' tests*

Some promising results have been obtained in an attempt to use the instrument for comparing the brittleness of biscuits of different moisture contents. The graphs show that with increase in moisture content the biscuits become, as is to be expected, less brittle. In the tests, the biscuits were placed over the holes in the lower strip of the cake holder and, as the sample platform rose, each one was pushed against a rounded plunger fitted in place of the compression block. Forces were built up till the biscuits snapped and these were recorded, the peak values representing the forces needed to break the biscuits.

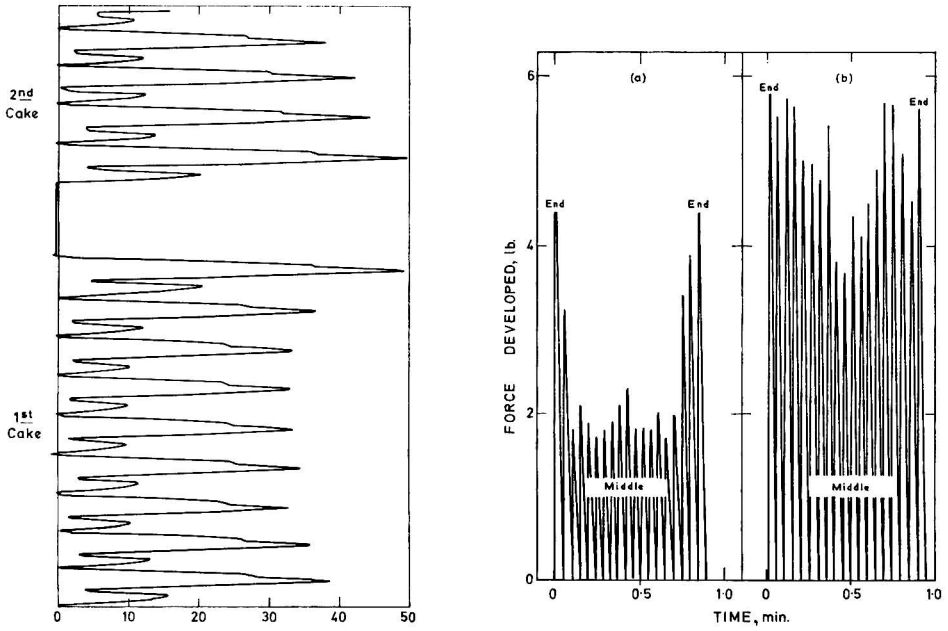

FIG. 9 (left).—*Recorder chart of shear and compressibility tests on Madeira cake*

(All slices of each cake, except crusts, were tested consecutively)
The force peaks are shown in pairs, the first (small one) being the shear and the second the compression

FIG. 10 (right).—*Rapid compressibility tests on bread*

(All slices of each loaf, except crusts, were tested consecutively)
(a) fresh (b) after 3 days

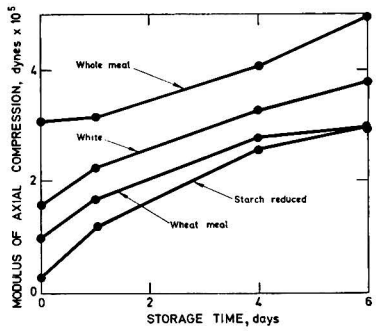

FIG. 11.—*Effect of storage of bread on firmness*

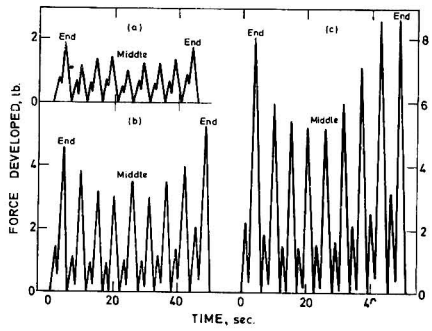

FIG. 12.—*Shear and compressibility tests on Madeira cake*
(a) fresh (b) after 1 day (c) after 3 days
(The force peaks are shown in pairs, the first being the shear and the second the compression
(All slices of each cake, except crusts, were tested consecutively)

The first tests, those on Rich Marie biscuits and shown in Fig. 14 indicate that 6 per minute is the most suitable testing rate, a faster speed outstripping the response of the recorder. This rate was used for the second tests on Coconut Crispies biscuits, the results of which are given in Fig. 15.

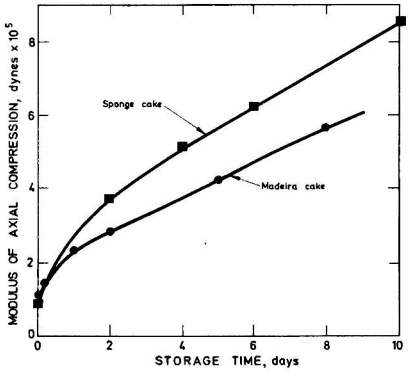

FIG. 13 (left)—*Effect of storage on firmness of cake*

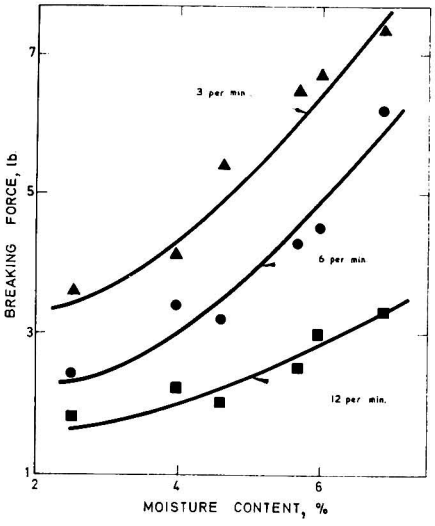

FIG. 14 (right).—*Snap tests at different testing rates on Rich Marie biscuits*

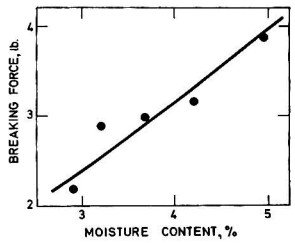

FIG. 15.—*Snap tests at 6 per minute on Coconut Crispies biscuits of different moisture contents*

Appendix

Modulus of axial compression: calculation

This modulus K is given as the compressive force per unit area divided by the change in volume per unit volume.

If F=the force in dynes,

A=the cross-sectional area of the compression block (cm^2),

D=the initial thickness of the bread (cm.) and d is the distance compressed (cm.).

$$\text{then } K = \frac{FD}{Ad}$$

Because the force F is measured in pounds then the expression becomes

$$K = \frac{FD}{Ad} \times 981 \times 454 \text{ (c.g.s. units)}$$

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Received 27 April, 1965

References

1 Cathcart, W. H., *Cereal Chem.*, 1940, **17**, 100
2 Bechtel, W. G., & Meisner, D. F., *Cereal Chem.*, 1954, **31**, 181
3 Bechtel, W. G., & Meisner, D. F., *Cereal Chem.*, 1954, **31**, 181
4 Brice, C. W., & Geddes, W. F., *Cereal Chem.*, 1949, **26**, 440
5 Kulp, K., Ponte, J. G., & Bechtel, W. G., *Cereal Chem.*, 1959, **36**, 228

THE FLAVOUR VOLATILES OF FATS AND FAT-CONTAINING FOODS. II*—A Gas Chromatographic investigation of Volatile Autoxidation Products from Sunflower Oil

By P. A. T. SWOBODA and C. H. LEA

Volatile compounds formed during the autoxidation of freshly refined sunflower oil at 37°, as well as those produced during subsequent heating of the oxidised oil, were concentrated by vacuum distillation and separated by gas chromatography.

Twenty-two individual aliphatic compounds (carbon chain length $\geqslant 5$) were identified by chromatographic retention on two stationary phases, supported by confirmatory physicochemical tests for functional group and carbon skeleton on isolated microgram amounts.

Semi-quantitative analysis, using a flame ionisation detector, demonstrated a marked preponderance of hexanal in the volatile products of low-temperature oxidation, whereas the much greater quantity of volatiles resulting from thermal decomposition of the first-formed hydroperoxides contained major proportions of the deca-2,4-dienals.

The formation of the identified compounds (saturated and unsaturated aldehydes, ketones, hydrocarbons and an alcohol) can be explained in large part by accepted mechanisms for the free radical decomposition of linoleate and oleate hydroperoxides, though it is not easy to account for the quantitative aspects.

Introduction

It is well established that saturated and unsaturated aldehydes and ketones constitute the main odorous decomposition products present in autoxidising edible fats and many investigations of such systems have employed chemical assay procedures specific for carbonyl compounds. For separation and determination of individual carbonylic compounds present, chromatography of the 2,4-dinitro-phenylhydrazones has generally been used.[1-3] Recently, gas–liquid chromatography has also been employed, but to a less extent and mainly qualitatively.[4-6]

To supplement previous studies,[7,8] in which the total volatile carbonyl content in a fat was determined, both before and after thermal decomposition of the peroxides present, we now describe the separation and semi-quantitative assay of individual volatile oxidation products by means of gas chromatography.

The investigation has been limited by the gas-chromatographic procedure employed to higher-molecular weight compounds, boiling around and above 100°, including however the regions where organoleptic properties are most marked. Characterisation of individual components by retention data has been supplemented, whenever a sufficient quantity of the compound could be isolated, by confirmatory tests to establish both functional group and carbon skeleton (Table I). Preliminary concentration or fractionation procedures have, however, been avoided, in order to minimise changes in the complex mixture of volatile compounds present, and sufficient material has not usually been available for i.r. or n.m.r. spectroscopy.

Table I

Identification of component volatiles

Gas chromatographic retention index data determined on

Squalane and *dinonyl phthalate* stationary phases by comparison with authentic compounds

Functional group established by

(i) Electron capture detector specific response
(ii) Spectrophotometric Assay of isolated fractions by u.v. absorption
2,4-Dinitrophenylhydrazones
Thiobarbituric acid test

Carbon skeleton established by

Vapour phase hydrogenation and deoxygenation of isolated components to yield hydrocarbon products which were identified by gas–liquid chromatography

* Part I: *J. Sci. Fd Agric.*, 1965, **16**, 18

Sunflower oil, shown by gas chromatography to be free from linolenate, was again used as experimental material, after preliminary purification to remove pre-formed oxidation products and controlled oxidation under mild conditions.

Experiment

Materials

Commercially refined sunflower oil was further purified by vacuum stripping to remove tocopherols, by treatment with silicic acid to remove non-volatile oxidation products and by steam deodorisation to remove volatile odorous substances and residual traces of solvent.[8] Autoxidation of the oil was carried out manometrically under oxygen at 37° to a level of approximately 100 μmole O_2 per g. of oil, followed by storage in completely filled, stoppered tubes at −20° or −80°, to minimise further change.

The purified oil (68·5% linoleate, 21% oleate) had a peroxide value of 0 and a total carbonyl content of 0·2 μmole/g.: after autoxidation it had a peroxide value of 97–100 μmole/g. and a total carbonyl content, after iodometric reduction of the peroxide, of 6 μmole/g. The volatile carbonyl content ranged from 0·2 to 17·6 μmole/g. according to the temperature of distillation used, the lower figure representing mainly the volatiles formed during oxidation and assayed with minimal decomposition of peroxide by low temperature distillation, and the higher value representing the volatile carbonyls present when all the peroxides had been thermally decomposed under vacuum at 200°.

Vacuum distillation apparatus

The apparatus used for recovery and collection of volatile compounds from autoxidised oils consisted of a simple retort still and trap evacuated by an oil diffusion-rotary pump system with Pirani gauge and argon inlet. The rate of evacuation was controlled by a two-way stopcock in the all-glass vacuum line which could either isolate the distillation flask from the pumping system or apply full capacity or restricted (via a capillary insert) pumping.[7,9] A second trap, cooled in liquid oxygen, protected the distillation system from contaminating volatiles which might diffuse back from the pumping system.

The retort still (Fig. 1) consisted of a 100-ml. round-bottomed flask with B 19/26 stoppered neck for sample addition, and a short wide side arm terminating in a glass to metal seal and 'Speedivac' R101 union (Edwards High Vacuum Ltd., Crawley). To this was connected the collection trap of concentric metal tube design (Swoboda, unpublished), of which the inner tube enters via a glass bead seal and terminates near the bottom of the trap with an electrical connection of platinum wire. In use, the inner tube is ohmically heated by applying a low voltage a.c. supply across the ends of the trap while the outer tube is cooled by immersion in liquid oxygen, thereby increasing the efficiency of collection. Since even silicone elastomer O rings can be a source of volatile impurities these were replaced in the R101 unions by Teflon cones (W. G. Pye & Co. Ltd., Cambridge).

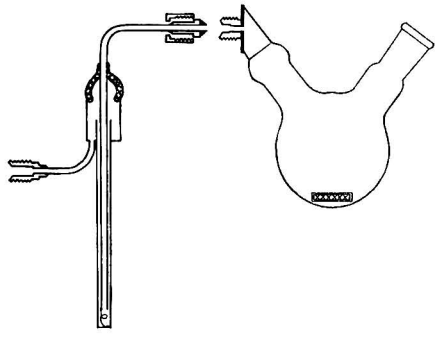

FIG. 1.—*Retort flask and concentric metal tube trap for distillation of volatiles from fats*

Vacuum distillation procedure

The procedure used was based on that previously developed to ensure complete removal of volatile carbonyls for chemical assay[7] though, inevitably, the distillation path had to be somewhat longer.

Low-temperature distillation, to remove volatiles formed during oxidation, was performed on 1–5 g. samples of oil heated at 75°. Initially, the apparatus was evacuated with the oil frozen in the bottom of the retort. Then, with the distillation system isolated, the most volatile components and dissolved gases were allowed to distil for 15 min. while the oil was heated and magnetically stirred at 100 r.p.m. Restricted pumping was then applied through the capillary for a further 30 min. followed by full capacity pumping for about 1½ h., after which the vacuum was released with argon.

High-temperature distillation was carried out on a 0·1–0·5 g. sample of oil. Initially, the apparatus was evacuated and the oil degassed. Then the retort flask was heated in a thermostated silicone oil bath at 210° with restricted pumping for 30 min. and thereafter for a further hour at full capacity.

Gas chromatography

Apparatus.—An Argon Ionisation Chromatograph (W. G. Pye & Co. Ltd., Cambridge) with 4 ft. × 4 mm. glass columns packed with squalane or dinonyl phthalate (15% on 80–100 mesh Celite) was used. To facilitate the transference of condensed volatiles from trap to column and the quantitative collection of components emerging from the detector, the necessary gas connections were made from stainless steel hypodermic needle tubing (Accles & Pollock Ltd., Birmingham).[9] Application of a low-voltage a.c. supply across the ends of these injection and collection adaptors heated them sufficiently to prevent loss by condensation.

When required, a second detector, flame ionisation or electron capture (W. G. Pye & Co. Ltd., Cambridge), was connected to the outlet of the collection adaptor. Simultaneous recording of analyses by two detectors could thus be achieved and comparison of the chromatograms obtained showed that there was no loss of resolution by peak broadening between the two detectors.

Although isothermal operation was preferred for the accurate determination of retention data for separated components, the time required to chromatograph mixtures of wide boiling point range could be reduced by programmed temperature operation. For this purpose the Argon Chromatograph was regulated by a modified Panchromatogram Temperature Programmer and Controller (W. G. Pye & Co. Ltd., Cambridge) operating at heating rates of 0·5 or 1° per minute between 50° and 110°: constant mass carrier gas flow was maintained by inserting a Flostat Minor III (G. A. Platon Ltd., Croydon) in the all metal gas supply line after the cylinder head gauge and molecular sieve drying tower.

Injection.—The concentric metal tube trap containing the vacuum distillate from the oil was connected, still cooled in liquid oxygen, to the remote injection adaptor by means of O ring sealed R101 unions, the flow of carrier gas being interrupted. The trap was then heated by replacing the liquid-oxygen coolant by an aluminium block thermostated at 210°, at the same time applying ohmic heating and starting a slow flow of argon gas. In this way the sample could be rapidly and completely volatilised and transferred to the column. After about half a minute, and before the emergence of an air peak, the carrier gas flow was increased to full.

When it was desired to incorporate one or more reference compounds into an analysis, a steel micropipette containing the marker mixture was inserted into a side arm of the cooled trap prior to its attachment to the remote injection adaptor.

Collection.—Alternative procedures were employed for collecting the eluate from the column according to whether the condensed component was to be re-volatilised or dissolved for further characterisation. In the former case, where the component was to be re-chromatographed on a stationary phase of different polarity or subjected to vapour phase hydrogenation, a metal concentric tube trap of similar design to that already described, but of smaller size, was used, with liquid oxygen as coolant. These traps are easier to use and give better recoveries than the multiple loop type originally employed.[9]

For spectrophotometric or organoleptic evaluation of the collected component, total trapping of the sample together with the argon carrier gas was achieved by cooling in liquid nitrogen.[10] The glass traps used for this purpose were modified from the original design in being of single piece construction, without a ground glass joint. To recover the trapped component the condensed argon was fractionally distilled away by transferring the trap to liquid oxygen, as previously described. Finally, the pressure in the still cooled trap was reduced to approximately 200 mm. Hg by controlled suction so that the sealed trap, on warming to room temperature, did not develop an excessive internal pressure with consequent loss of sample when unsealed for addition of solvent or reagent.

Vapour-phase hydrogenation

The carbon skeletons of components isolated from the analytical gas chromatograph were characterised by catalytic hydrogenation and deoxygenation, with identification of the hydrocarbon products by gas chromatography. Although this method is generally applicable to organic compounds containing oxygen, nitrogen, sulphur and even halogens, scission of the carbon–carbon bond next to a functional group is known to occur in some cases, producing a mixture of hydrocarbons of reduced chain length.[11, 12] A platinum catalyst at 215° was found to cause less cracking than palladium or nickel, but the relative proportions of the hydrocarbons formed varied with the batch and age of the catalyst. Although a lower temperature of operation reduced the degree of chain scission, it also increased adsorption in the catalyst tube, leading to contamination in subsequent analyses.

In operation, the catalytic hydrogenator was connected in series to a product collection trap and gas chromatographic column with flame ionisation detector. The hydrogenator consisted of a stainless steel tube (2 mm. bore) containing a 6 in. packing of 5% platinum on 60–80 mesh silica (Johnson, Matthey & Co. London), thermostatically maintained at 215° in a muffle furnace. Liquid or vapour samples were injected into the tube by syringe through a silicone rubber septum: for condensates in concentric tube traps, a hypodermic needle was attached to the latter and the sample volatilised by a hot air blower and flushed into the reaction tube by switching the hydrogen flow through the trap. The purified hydrogen (40 ml./min.) used for the catalytic hydrogenation served also as carrier gas for the subsequent chromatographic separation.

To avoid tailing of the products from the catalytic reaction tube affecting the resolution of the gas chromatogram a seven minute collection period after injection was adopted. The contents of the product collection trap were then re-volatilised on to the chromatographic column, which consisted of a 5 ft. length of 2 mm. bore stainless steel tubing packed with 10% squalane on 80–100 mesh Celite, thermostated in a water bath at a temperature appropriate to the separation of the expected hydrocarbons.

Spectrophotometric assay

Since 1μ mole of a compound with $\epsilon = 10{,}000$ has an absorbance of unity when dissolved in 10 ml. of solution, the sensitivity of many spectrophotometric assays is adequate for the identification of functional groups in major components isolated from analytical gas chromatographic columns. When, however, smaller amounts are to be detected it has been found more satisfactory to trap sequentially for equal time intervals, rather than for individual peaks, dividing an entire programmed temperature chromatogram into perhaps 60 fractions. A presentation of the spectrophotometric assays in histogram form then indicates when a detected component emerges and also defines the extent of any overlapping and tailing of major components.

The following assays have been performed using a Beckman DK2 recording spectrophotometer.

(1) *Direct ultra-violet absorption.*—The contents of each total trap were dissolved in a minimum of 4 ml. of cyclohexane (spectroscopically pure) and the spectrum recorded between 210 and 360 mμ.

(2) *Absorption of the 2,4-dinitrophenylhydrazine derivatives.*—The cyclohexane solution of

the fraction was passed through a reaction column of Celite impregnated with 2,4-dinitrophenylhydrazine, phosphoric acid and water, to effect rapid and quantitative conversion of carbonyl compounds to their DNPH derivatives.[2, 13] These were then eluted in 10 ml. of hexane, leaving excess reagent on the column, and the spectrum recorded between 320 and 470 mμ, to obtain the absorbance maximum in neutral solution. The solvent was then removed on a rotary evaporator and replaced by 0·1N-sodium hydroxide in chloroform–ethanol (1:9, v/v), for determination of the spectrum between 400 and 580 mμ under alkaline conditions. Bathochromic shifts and increasing absorbance are observed for carbonylic compounds with increasing conjugated unsaturation.

(3) *Thiobarbituric acid (TBA) reaction.*—The contents of each total trap were dissolved in a minimum of 4 ml. of TBA–ferric iron reagent, prepared by dissolving 0·72 g. of TBA and 3·24 mg. of anhydrous ferric chloride in 200 ml. of 50% aqueous acetic acid.[14] After heating in a water bath at 70° for 30 min. and cooling, the absorption spectrum was recorded between 410 and 590 mμ. Ketones give no colour. Aldehydes, irrespective of structure give a yellow colour ($\lambda_{max} \simeq 450$ mμ), and a second red colour ($\lambda_{max} \simeq 525$) is given by compounds such as conjugated dienals which are presumably partly converted to malonaldehyde under the conditions of the test. Comparative studies with authentic compounds established the reference spectrum for each structural type and showed that all three spectrophotometric assays had sensitivities of the same order of magnitude.

Results

The gas chromatogram of the volatiles isolated from autoxidised sunflower oil proved to be very complex. Fig. 2 shows the components recovered from a high temperature distillation during which all peroxides were decomposed under vacuum. The chromatogram was obtained on squalane (column C) temperature programmed at 0·5° per minute between 50° and 110°. Although over 40 components are discernable, only four are present in major amounts, namely hexanal, hept-2-enal and the two isomeric deca-2,4-dienals. The 22 numbered peaks have been identified.

In order to determine chromatographic retention data more precisely and express them in the form of Kováts Retention Indices on a logarithmic scale relative to the homologous n-alkanes,[9, 15] replicate isothermal chromatograms were run, with and without added n-alkane markers, for both high and low temperature distillates: the columns used at 75° were squalane (column A) and dinonyl phthalate (column B). For aliphatic compounds the Kováts Retention Index can be considered as being the sum of two additive terms, one deriving from the number of carbon atoms in the molecule and the other from the functional group. Thus, members of a homologous series have retention indices which differ by one hundred units for each additional methylene group, whilst different chemical compounds of the same chain length differ according to the contribution of the particular functional group.[9] A number of authentic reference compounds were chromatographed isothermally on the two stationary phases to determine the

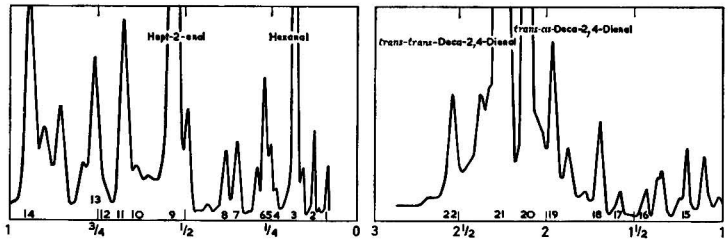

FIG. 2.—*Programmed temperature chromatogram of volatiles recovered from a high temperature distillate of autoxidised sunflower oil*

The numbered peaks are identified in Table II

functional retention indices for the alkanal, 2-enal and 2,4-dienal classes, thus permitting identification for all members of these series though not all were available for reference.

However, with a very complicated chromatogram it is not always easy to correlate the retention of a particular component on two stationary phases, nor to allow for overlapping peaks. Individual peaks emerging from programmed temperature runs on the squalane column C were therefore re-chromatographed isothermally on dinonyl phthalate, to establish their identity. The retention indices for the compounds identified are included in Table II. Under isothermal conditions at 75° a tenfold increase in retention volume corresponded to an increase of 274 retention index units for squalane and 287 units for dinonyl phthalate. The retention index units quoted for programmed temperature operation are determined from curved carbon number plots and are only approximate, though reproducible. The divergence of these values from the isothermal results may be due partly to this, and partly to a difference in polarity between the two squalane columns used (A and C).

A semi-quantitative analysis is also included in Table II, expressing as weight percentage the peak areas derived from programmed temperature chromatograms: for this purpose the peaks were cut out from the recorder chart and weighed, with due allowance for any base line changes. A flame ionisation detector was employed because of its wide linear dynamic range and insensitivity to water vapour. The known variation of response of this detector to different compounds and the difficulty of ensuring completely quantitative recoveries on distillation and

Table II

Semi-quantitative analysis and chromatographic retention data of compounds identified in the volatiles isolated from autoxidised sunflower oil

Peak No.	Identified component	Approximate % composition of volatiles, isolated by distillation at		Isothermal retention index at 75° on		Approximate* programmed temperature retention index on
		210°	75°	Squalane Column A	Dinonyl phthalate Column B	Squalane Column C
1	Pentanal	0·7	4	648	787	640
2	Heptane	0·8	0·1	696	700	700
3	Hexanal	12	50	755	890	755
4	Oct-1-ene	0·2	0·1	782	800	785
5	Hex-2-enal	0·3	0·1	811	977	790
6	Octane	1	0·5	801	800	800
7	Heptan-2-one	0·6	0·5	844	986	835
8	Heptanal	0·7	0·5	857	992	845
9	Hept-2-enal	10	4	909	1075	900
10	Oct-1-en-3-one	0·4	0·1	921	1074	925
11	Oct-1-en-3-ol	2	2	933	1111	935
12	Octanal	1	0·6	958	1093	
13	Hepta-2,4-dienal				1156	955
14	Oct-2-enal	2	4	1009	1179	1000
15	Nonanal	0·8	1	1056	1194	1055
16	Non-2-enal	0·3	2	1108	1280	1100
17	*trans,cis*-Nona-2,4-dienal	0·3	0·5	1137	1311	1130
18	*trans,trans*-Nona-2,4-dienal	0·8	0·5	1159	1359	1150
19	Dec-2-enal	2	1	1208	1380	1200
20	*trans,cis*-Deca-2,4-dienal	15	1	1234	1418	1230
21	*trans,trans*-Deca-2,4-dienal	35	1	1258	1452	1260
22	Undec-2-enal	2	1	1311	1479	1300

* Values rounded off to nearest five units

chromatography limit the accuracy of the analysis, the results from the low temperature distillation being the less precise of the two because the total yield of volatiles was small. The figures have therefore been rounded off to one significant figure and represent the percentage of each identified component in the total mixture of volatiles observed on the chromatogram.

Spectrophotometric data for the four major components and three minor ones are given in Table III: for many of the minor components the quantities available were too small for absorption maxima to be determined accurately. The assignation of *trans,cis* and *trans,trans* configurations to the 2,4-dienals separated is based on the chromatographic retentions described by Hoffmann & Keppler.[5,16]

Table III

Spectophotometric data for some isolated carbonyl compounds

Peak No.	Identified component	Ultra-violet	Dinitrophenylhydrazone		Thiobarbituric acid absorbance	
		λ_{max} in cyclohexane mμ	λ_{max} in neutral solution, mμ	λ_{max} in alkaline solution, mμ	at 450 mμ	at 525 mμ
1	Pentanal	—	337	430	+	—
3	Hexanal	—	340	428	+	—
9	Hept-2-enal	216	359	455	+	—
14	Oct-2-enal	217	358	455	+	—
19	Dec-2-enal	219	357	452	+	—
20	*trans,cis*-Deca-2,4-dienal	270	375	478	+	+
21	*trans,trans*-Deca-2,4-dienal	268	375	477	+	+

The heptanone found was probably methyl pentyl ketone and the chromatographic retention observed agreed with that of the authentic compound. The occurrence of heptan-2-one has been reported in reverted soya-bean oil[3] and in oxidised butterfat.[4] However the isomeric heptan-4-one has also been reported as a product of linoleate oxidation.[17]

The electron capture detector confirmed the presence of small amounts of hepta-2,4-dienal and of *trans,cis*- and *trans,trans*-nona- and deca-2,4-dienals. This detector also gave a strong response with oct-1-en-3-one, presumably because of its vinyl ketone structure. Both oct-1-en-3-ol and the corresponding ketone were prepared synthetically by the procedure of Crabalona[18] and Brown & Garg[19] and the identifications confirmed by coincidence of retention. Moreover oct-1-en-3-ol has such a characteristic and penetrating fungal-earthy odour, suggestive of mushrooms, that its emergence from the gas chromatogram can be established by smell.

The hydrocarbons heptane and octane coincided with the authentic compound on both columns, as also did oct-1-ene: the latter was not separated from octane on dinonyl phthalate.

In all chromatographic separations of complex mixtures overlapping of some of the components is liable to occur. Octanal and hepta-2,4-dienal were the only pair so overlapping when the sunflower volatiles were temperature programmed on squalane and their presence was detected on re-chromatography on dinonyl phthalate. No evidence was obtained for the presence of any appreciable quantity of decanal or undecanal, which would have run with the nonadienal or decadienal peaks, or for any of the lower dienals.

Discussion

Although the free-radical chain reaction mechanism for the autoxidation of olefins such as the unsaturated fatty acids has been well authenticated, there is little direct evidence about the mechanism of the subsequent scission of the hydroperoxides to the volatile decomposition products which are organoleptically important.[20] The reaction usually postulated is the thermal breakdown of the hydroperoxide into hydroxy and alkoxy free-radicals, the latter then undergoing scission of the carbon–carbon bond adjacent to the carbonyl group (Fig. 3). Of the resulting fragments one is a carbonyl compound whilst the other is an alkyl (type A scission) or a vinyl (type B scission) free-radical. Frankel *et al.*[21] have suggested that the alkyl free-radical might

yield a hydrocarbon by hydrogen abstraction or an alcohol by reaction with the hydroxyl free-radical. Reaction with oxygen would give a primary hydroperoxy radical. However, these free radical fragments could react in so many ways that it is not possible, *a priori*, to predict a major volatile product.

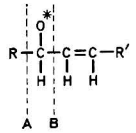

FIG. 3.—*Postulated types of chain scission (A and B) for the alkoxy radical derived from unsaturated fatty acid hydroperoxides*

R and R¹ may be either the methyl or carboxyl end of the fatty acid

In the case of oleic acid, the four known isomeric hydroperoxides, the 8-, 9-, 10- and 11-hydroperoxy-monoenes, would be expected to yield the volatile carbonyl compounds undec-2-enal and dec-2-enal by type A and nonanal and octanal by type B scission, respectively.

During the much faster autoxidation of the 1,4-diene system of linoleate the major products are the conjugated 9- and 13-hydroperoxides. The former would cleave by type A scission to yield deca-2,4-dienal and the latter hexanal by type B scission. As minor products of autoxidation, linoleate might be expected to form five unconjugated hydroperoxides. Four of these would arise by monoene type oxidation initiated by hydrogen abstraction from the 14- and 8-methylene groups. From the former the 14- and 12-hydroperoxides would cleave to give pentanal (type B) and hept-2-enal (type A) respectively.[22] The analogous 10- and 8-hydroperoxides would give non-3-enal (type B) and undec-2,5-dienal (type A). Of all the carbonyl compounds predicted by the postulated reaction scheme for monoene and 1,4-diene oxidation and cleavage, only the last two compounds have not yet been experimentally identified. Finally, the unconjugated 11-hydroperoxy-9,12-diene, the existence of which has still not been experimentally demonstrated, has in the past been suggested as the precursor of oct-2-enal by type B scission from linoleate.

Suggestions as to the mechanism of formation of the other volatile compounds derived from oxidised sunflower oil are even more speculative. Thus the observed hydrocarbons, octane and heptane are the longest n-alkanes that could arise via a free alkyl radical by type A scission of an unsaturated hydroperoxide, the 10- and 11-hydroperoxyoleate. Alternative mechanisms have been proposed for the formation from linoleate of oct-1-en-3-one,[23, 24] and oct-1-en-3-ol.[22, 25] To account for the homologous dienals it might be necessary to postulate the presence of minor amounts of positional isomers of linoleic acid, as was so elegantly confirmed in a recent study of the flavour potency and origin of hept-4-enal in butter fat.[26, 27]

Certain quantitative aspects are difficult to reconcile with the mechanism here discussed. Thus, whilst hexanal is the major volatile compound formed during low temperature autoxidation, deca-2,4-dienal predominates on thermal decomposition; and yet both arise from positional isomers of conjugated linoleate hydroperoxide. This difference can best be explained by the selective further oxidation of dienals during autoxidation.[28] Again, the high yield of hept-2-enal cannot be accounted for solely by the scission of 12-hydroperoxy linoleate, which could be only a minor oxidation product.

Although some earlier papers reported hexanal, oct-2-enal and deca-2,4-dienal as the predominant volatiles from oxidised linoleate or linoleate containing oils,[29, 30] later workers using more refined techniques have described more complex mixtures, depending in part on the degree and conditions of oxidation and on the unsaturation of the oil. Our results agree in the main with those of Gaddis *et al.* who analysed the carbonyl compounds by paper chromatography of their 2,4-dinitrophenylhydrazine derivatives.[1] They also showed that in fats containing linoleate and oleate, the major carbonylic compound formed during oxidation at low temperature was hexanal, and that on heating a much greater yield of volatile carbonyls was obtained containing predominantly deca-2,4-dienal. In contrast to earlier work, we both found hept-2-enal as a major mono-unsaturated component. However, neither pentanal nor heptanal

were observed by Gaddis *et al.*, though El-Negoumy *et al.*[6] who reported hexanal, hept-2-enal and oct-2-enal as the major volatile products from linoleate autoxidation at 50°, also identified pentanal and heptanal as well as deca-2,4-dienal among the minor constituents.

Effect on flavour

As yet, the flavour potency of all the volatile oxidation products as individuals has not been investigated, though some information is available.[31–33] Mutual interaction effects would obviously make a full investigation extremely complicated. Even with the unfractionated mixture of volatile oxidation products, organoleptic response depends to a marked extent in intensity and character on the medium in which it is diluted for tasting (Table IV).

Table IV

Flavour threshold of the volatile carbonyls distilled from sunflower oil oxidised to a peroxide value of 100 μmoles/g.

Dispersed for tasting in	Threshold concentration (50% detection method)	
	CO, μmole/litre	p.p.m.*
Volatiles removed in 1 h. at 50° (0·2 μmole/g. oil)		
Water	0·03	0·03
Separated milk	0·2	2
Separated milk + 5% oil	0·5	5
Separated milk + 10% oil	1·0	10
Oil	3·4	34
Volatiles removed in 1 h. at 200° (17·6 μmoles/g. oil)		
Water	0·008	0·08
Separated milk	0·09	0·9
Oil	1·7	17

* Assuming an average mol. wt. of 100 (hexanal)

Because of its large concentration, hexanal must make an appreciable contribution to the rancid odour of the low temperature autoxidised oil and deca-2,4-dienal is probably the major contributor to the ' deep fried-oily ' aroma of the high-temperature distillate.[34] Other identified components known to possess strong and characteristic flavour potencies are oct-1-en-3-one which is said to be largely responsible for oxidised metallic off-flavour in dairy products,[23, 24] and oct-1-en-3-ol which has a mushroom-like odour.[22, 25] Observations on the flavour contribution of some of the other carbonylic compounds here identified have been described in investigations of fishy, painty and tallowy taints in butterfat.[4]

Acknowledgments

Thanks are due to H. A. F. Jackson and P. J. Halls for much of the experimental work.

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Received 27 April, 1965

References

1 Gaddis, A. M., Ellis, R., & Currie, G. T., *J. Amer. Oil Chem. Soc.*, 1961, **38**, 371
2 Schwartz, D. P., Haller, H. S., & Keeney, M., *Analyt. Chem.*, 1963, **35**, 2191
3 Mookherje, B. D., & Chang, S. S., *J. Amer. Oil Chem. Soc.*, 1963, **40**, 232
4 Forss, D. A., Dunstone, E. A., & Stark, W., *J. Dairy Res.*, 1960, **27**, 211, 373, 381
5 Hoffmann, G., *J. Amer. Oil Chem. Soc.*, 1961, **38**, 31
6 El-Negoumy, A. M., De Puchal, M. S., & Hammond, E. G., *J. Dairy Sci.*, 1962, **45**, 311
7 Lea, C. H., & Swoboda, P. A. T., *J. Sci. Fd Agric.*, 1962, **13**, 148
8 Lea, C. H., & Hobson-Frohock, A., *J. Sci. Fd Agric.*, 1965, **16**, 18
9 Swoboda, P. A. T., Gas Chromatography, 1962 p. 273 (Proc. 4th Int. Symposium) (London: Butterworths)
10 Swoboda, P. A. T., *Nature, Lond.*, 1963, **199**, 31
11 Thompson, C. J., Coleman, H. J., Hopkins, R. L., Ward, C. C., & Rall, H. T., *Analyt. Chem.*, 1960, **32**, 424, 1762; 1962, **34**, 151, 154

References (*cont.*)

[12] Beroza, M., & Sarmiento, R., *Analyt. Chem.*, 1962, **34**, 1801; 1963, **35**, 1353
[13] Schwartz, D. P., & Parks, O. W., *Analyt. Chem.*, 1961, **33**, 1396
[14] Täufel, K., & Zimmermann, R., *Fette u. Seifen*, 1961, **63**, 226
[15] Ettre, L. S., *Analyt. Chem.* 1964, **36**, No. 8, 31A
[16] Hoffmann, G., & Keppler, J. G., *Nature, Lond.*, 1960, **185**, 310
[17] Schepartz, A. I., & Daubert, B. F., *J. Amer. Oil. Chem. Soc.*, 1950, **27**, 367
[18] Crabalona, L., *Bull. Soc. chim. Fr.*, 1944, **11**, 67
[19] Brown, H. C., & Garg, C. P., *J. Amer. chem. Soc.*, 1961, **83**, 2952
[20] Schultz, H. W., Day, E. A., & Sinnhuber, R. O., 'Symposium on Foods: Lipids and their Oxidation.' 1962 (Connecticut: A.V.I. Pub. Co.)
[21] Frankel, E. N., Nowakowska, J., & Evans, C. D., *J. Amer. Oil Chem. Soc.*, 1961, **38**, 161
[22] Hoffman, G., *J. Amer. Oil Chem. Soc.*, 1962, **39**, 439
[23] Stark, W., & Forss, D. A., *J. Dairy Res.*, 1962, **29**, 173
[24] Hammond, E. G., & Hill, F. D., *J. Amer. Oil Chem. Soc.*, 1964, **41**, 180
[25] Stark, W., & Forss, D. A., *J. Dairy Res.*, 1964, **31**, 253
[26] Begemann, P. H., & Koster, J. C., *Nature, Lond.*, 1964, **202**, 552
[27] De Jong, K., & Van Der Wel, H., *Nature, Lond.*, 1964, **202**, 553
[28] Day, E. A., & Lillard, D. A., *J. Amer. Oil Chem. Soc.*, 1964, **41**, 549
[29] Swift, C. E., O'Connor, R. T., Brown, L. E., & Dollear, F. G., *J. Amer. Oil Chem. Soc.*, 1949, **26**, 297
[30] Badings, H. T., *J. Amer. Oil Chem. Soc.*, 1959, **36**, 648
[31] Lea, C. H., & Swoboda, P. A. T., *Chem. & Ind.*, 1958, p. 1289
[32] Day, E. A., Lillard, D. A. & Montgomery, M. W., *J. Dairy Sci.*, 1963, **46**, 291
[33] Meijboom, P. W., *J. Amer. Oil Chem. Soc.*, 1964, **41**, 326
[34] Patton, S., Barnes, I. J., & Evans, L. E., *J. Amer. Oil Chem. Soc.*, 1959, **36**, 280

NOTE ON A PEPSIN-NORMAL ACID DIGESTION FOR THE DETERMINATION OF FIBROUS RESIDUE IN HERBAGE

By G. ap GRIFFITH and D. I. H. JONES

In the N-acid fibre determination, a preliminary digestion with pepsin and hydrochloric acid reduces further the N-content of the fibre and further improves correlation with digestibility.

In a previous paper[1] it was shown that increasing the time of digestion of herbage with N-sulphuric acid effected a substantial improvement in the correlation of fibre content with the *in vivo* digestibility of the herbage. This improvement appeared to be due to a reduction in the nitrogen content of the residue caused by the longer digestion. The 3-h. digestion removed approximately the same amount of nitrogen as would a pepsin–hydrochloric acid digestion of the original herbage.

Further study has revealed that if the N-acid digestion is preceded by a pepsin–hydrochloric acid digestion, a further reduction in the nitrogen content of the fibre is achieved (Table I).

Table I

Nitrogen remaining in the fibre residue expressed as % of original herbage

	Pepsin/hydrochloric acid digestion	N-Sulphuric acid digestion	Pepsin/hydrochloric acid followed by N-acid digestion
Sample A	0·42	0·53	0·15
Sample B	0·33	0·30	0·12

Following this observation, a preliminary pepsin–hydrochloric digestion was then applied to the N-acid fibre determination on 32 samples of herbage of known *in vivo* and *in vitro* dry-matter digestibility. Correlation coefficients of the results obtained are summarised in Table II.

Table II

Correlation coefficients of digestibility with fibre content

	In vitro digestibility	Pepsin/hydrochloric acid on N-acid fibre	Normal acid fibre
In vivo digestibility	+·917***	−·892***	−·855***
In vitro digestibility		−·942***	−·909***
Pepsin/HCl + normal acid fibre			+·969***

***P = 0·001

It appears from these results that a further improvement in correlation is effected by the additional pepsin digestion. The modification adds little to the manipulations required, although it lengthens the time taken for a single determination.

The method as now used is as follows: 1 g. of the dried ground herbage is extracted with ethanol–benzene and transferred to a 100-ml. tube with 50 ml. of pepsin–hydrochloric acid solution (0·2% pepsin in 0·1 N-hydrochloric acid). The tube is left in a water bath at 40° for 24 h., with occasional gentle agitation. The contents are filtered through a sintered glass crucible and washed. The residue is washed into a flask with N-sulphuric acid and digestion is continued as previously described.[1]

This technique has recently been applied to the study of a number of varieties of different grass species. The fibre values have proved to be in close agreement with the *in vitro* digestibility values in the ranking of varieties. The correlation between the two is less close as the hay stage is approached. At this period the fibre percentage levels off, whilst the *in vitro* digestibility continues to fall. This may imply that there is a change in the nature of the structural constituents rather than an increase in fibre at the later stage of maturity. Unlike the residue from *in vitro* or *in vivo* digestion, the fibre residue consists largely of cellulose, xylan being largely soluble in normal sulphuric acid. Since the xylan is less digestible,[2] an increase in its proportion with maturity will reduce digestibility without increasing fibre percentage. The application of the normal acid fibre method, with or without pepsin digestion, to the evaluation of hays may, therefore, need separate consideration.

Acknowledgment

The statistical help of Dr. E. L. Breese is acknowledged.

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Received 4 May, 1965

References

[1] ap Griffith, G., & Jones, D. I. H., *J. Sci. Fd Agric.*, 1963, **14**, 380

[2] Waite, R., Johnston, M. J., & Armstrong, D. G., *Proc. Nutr. Soc.*, 1963, **22**, XXIV

AIR–WATER PARTITION COEFFICIENTS OF SOME ALDEHYDES

By RON G. BUTTERY, D. G. GUADAGNI and SUMIKO OKANO

The air–water partition coefficients of a series of aldehydes from propanal to decanal in dilute aqueous solution have been measured by gas chromatography. The coefficients did not vary appreciably over the whole series, in contrast to the decreasing vapour pressures of the pure solutes.

Introduction

The aroma of a food is due to the small concentration of volatile compounds in the atmosphere above the food. The concentration of a compound in the atmosphere is controlled by a partition of the volatile compound between the food itself and the air above the food. Since most foods contain 50–90% of water it might be expected that this would largely be an air–water partition. A study has therefore been made of the air–water partition coefficients of some normal homologues from C_3 to C_{10} at 25° and atmospheric pressure.

The concentrations of the aldehydes in the air- and the water-phase were determined with a gas–liquid chromatography (GLC) apparatus equipped with dual flame ionisation detectors. Solutions containing 5 g. of aldehyde per 10^6 g. of water were used. Such solutions are so close to infinite dilution that Henry's law should hold and the partition coefficients be constant at constant temperature and pressure. Burnett & Swoboda[1] showed this to be true for water solutions of acetone and ethanol in the range of about 1 to 1000 p.p.m., and it has been demonstrated in this laboratory for water solutions of hexanal in the range of 0·02 to 1 p.p.m.[2].

Experimental

Materials

Aldehydes were obtained from reliable commercial sources or synthesised by well established methods. Each compound was purified by GLC separation and purity was checked by refractive index and GLC. Water was distilled and then boiled to remove air and volatile impurities.

Apparatus

The GLC apparatus was a Perkin-Elmer Model 800 with dual flame ionisation detection. The dual columns were 10 ft. by $\frac{1}{8}$ in. o.d. stainless steel, packed with 20% Apiezon L on 60–80 mesh Chromosorb W, and operated at temperatures ranging from 50° for propanal to 120° for decanal. Nitrogen saturated with water was the carrier gas; the flow rate was 30 ml. per min.

Syringes were of the conventional glass cylinder and barrel type, chosen to be sufficiently gas tight. All apparatus was washed with detergent, rinsed thoroughly with distilled water and dried at 100° overnight before use.

Method

Solutions of the aldehyde were made up at a concentration of 5 p.p.m. (w/w) in boiled distilled water at room temperature.

For sampling the vapour, a 6-oz. Teflon bottle was half filled with the solution to be tested and set aside for 15 min. at 25° with occasional shaking. The needle of a 2-ml. hypodermic was then pushed through the cap of the bottle, and the headspace gas forced into the syringe up to the 2-ml. mark by squeezing the bottle. The sample was then injected directly into the GLC unit.

The solution itself was injected in 10·0 μl. samples taken out of the bottle used for the vapour samples. Injection temperature was 150° to ensure rapid vaporisation of the solution.

Peak areas were obtained by multiplying the peak height by the peak width at $\frac{1}{2}$ peak height. The partition coefficients given in Table I are averages of at least five determinations.

Table I

Partition coefficients of C_3–C_{10} n-alkanals between air and water at 25° and atmospheric pressure

Compound	Partition coefficient[a]
Propanal	$54 \pm 7^b \times 10^{-4}$
Butanal	63 ± 12 ,,
Pentanal	69 ± 5 ,,
Hexanal	69 ± 13 ,,
Heptanal	67 ± 10 ,,
Octanal	54 ± 8 ,,
Nonanal	57 ± 14 ,,
Decanal	24 ± 12 ,,

[a] Weight of aldehyde per ml. of air/weight of aldehyde per ml. of water.
[b] Standard deviation, calculated from 5 or more determinations

Results and discussion

The partition coefficients were determined as k=(weight of aldehyde per ml. of air)/(weight of aldehyde per ml. of water) at 25° and 1 atmosphere pressure. The injection of both solution and vapour allows us to cancel out the GLC weight-to-area conversion factors and simplify the calculation to:

k=(*peak area for vapour*) × (*volume of solution injected*)/(*peak area for solution*) × (*volume of vapour injected*

Table I lists the partition coefficients found for the aldehydes examined. They do not range widely, in fact, they are the same within experimental error except that for decanal which is significantly lower.

Burnett[3] and Nawar & Fagerson[4] have reported similar lack of variation for air–water partition coefficients of a C_1–C_4 series of alchocols and a C_3–C_7 series of 2-alkanones, respectively.

The air/water partition coefficients have considerable significance in any comparison of the effectiveness of different aldehydes as odorants where comparison is made in dilute water solutions (cf. Lea & Swoboda[5]). The difference in the number of molecules reaching the olfactory sensors may not be as great as one might expect considering the vapour pressures of the pure substances.

When dealing in quantitative analysis at very low concentrations, there is some doubt whether the adsorption of compounds on the glass wall of the syringe can be neglected. The degree of adsorption of propanal and hexanal above aqueous solutions of 10 p.p.m. when measured by a method previously described, was found to be less than 2%. It is presumed that other aldehydes would have an equally negligible adsorption.

Acknowledgments

The authors wish to thank the Instant Potato Granule Manufacturers Association and the U.S. Brewers Association for partial support of this work.

(Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Dept. of Agriculture to the exclusion of others that may be suitable.)

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Received 12 April, 1965; amended manuscript 19 July, 1965

References

[1] Burnett, M. G., & Swoboda, P. A. T., *Analyt. Chem.*, 1962, **34**, 1162
[2] Buttery, R. G., & Teranishi, R., *J. agric. Fd. Chem.*, 1963, **11**, 504
[3] Burnett, M. G., *Analyt. Chem.*, 1963, **35**, 1567
[4] Nawar, W. W., & Fagerson, I. S., *Food Technol.*, 1962, **16**, (11), 107
[5] Lea, C. H., & Swoboda, P. A. T., *Chem. & Ind.*, 1958, P. 1289

J. Sci. Fd Agric., 1965, Vol. 16, November

JOURNAL OF THE SCIENCE OF FOOD AND AGRICULTURE

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Printed in Great Britain by Unwin Brothers, Ltd., Woking and London (B4587)