mayonnaise-like emulsions and produced high levels of foam. At pH 6.7, 8.2, and the two-step pH treatments, water and low salt mixtures resulted in soluble proteins having similar electrophoretic properties. These gels showed that suspensions with 1.0 M NaCl had lower amounts of the nonarachin proteins in region 2.5–5.5 cm which could be related to the poor emulsions from these preparations. Moreover, the arachin components were diffuse and difficult to discern in these gels. No specific protein properties detectable by gel electrophoresis could be related to the unique ability of the water suspension subjected to the two-step pH adjustment of 6.7 to 4.0 to 8.2 to form a mayonnaise-like emulsion.

CONCLUSIONS

In meat systems, the quantity as well as the quality of aqueous salt-soluble protein is an excellent measure of certain functional properties of these products in foods (Saffle, 1968). Our data suggest that predicting the functional properties of defatted peanut meals cannot be done on the basis of proteins alone. Water, proteins, carbohydrates, oil, and a number of unknown constituents in oilseed meals evidently interact differently than in meat systems depending on pH and salt levels (Lin et al., 1974; Cherry et al., 1975; McWatters and Cherry, 1975). Studies on interactions of processing conditions and protein composition such as those explored in this study should lead to an understanding of their effects on the functionality of peanut protein products. Processors are now beginning to develop some of the new and expanded areas for peanut utilization dealing with high protein meals, flours, concentrates, and isolates. Furthermore, the behavior of specific peanut components must receive more research emphasis if the potential of peanut products as functional ingredients is to be fully realized. One example of the significance of such studies is presented in this paper in that adjusting the pH of peanut meal suspensions in water from 6.7 to 4.0 to 8.2 (or water and low salt mixture from pH 6.7 to 1.5) dramatically improved their functional behavior in emulsion formation.

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An Enzymic Assay for Acetate Fruit Juices and Wines

Leo P. McCloskey

A rapid enzymic method for the determination of acetate anion in juices is described. It utilizes three coupled enzyme mediated reactions so that the spectrophotometric determination of NADH is stoichiometrically related to acetate anion levels. The method is described for use with vegetable and fruit juices, and wine. The recovery of acetate anion was 97–102%, with standard deviations as good as 0.25 mg/100 ml (2.5 ppm) in an apple juice with 22 mg/100 ml of acetate anion. The procedure was compared to a steam distillation procedure, showing that the enzymic assay was vastly superior as to accuracy and recovery of acetate. The procedure should be of advantage in agricultural areas involving the processing of fruits and their products, including fermented beverages. One operator can perform 200 assays per day manually and more with some automation.
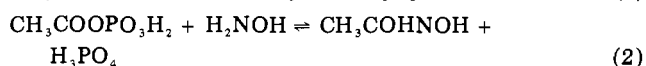
There are various enzymatic assays for the carboxylic acids, including acetic acid, described in the literature

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(Bergmeyer, 1963, 1974b; Holz and Bergmeyer, 1970; Postel and MacCagnan, 1971). The availability of enzymes commercially at reasonable prices is a limiting factor in the feasibility of the use of some of these assays (Bergmeyer, 1974a; Lundquist et al., 1961).
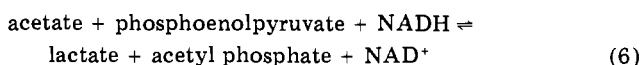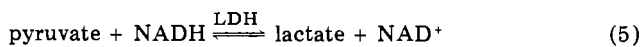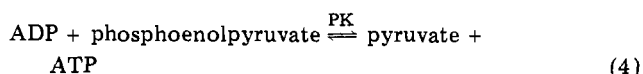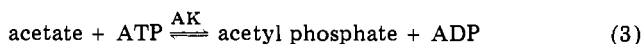
A practical and popular method used for the measurement of acetate in biological fluids is that of Rose

(1962). Various applications of this method have been made (Bergmeyer, 1963; Rose, 1955). The assay is based on reactions 1 and 2.

$$CH_3COOH + ATP \xrightleftharpoons{AK} CH_3COOPO_3H_2 + ADP \qquad (1)$$

$$CH_3COOPO_3H_2 + H_2NOH \rightleftharpoons CH_3COHNOH + H_3PO_4 \qquad (2)$$

The procedure, as described by Bergmeyer (1963), was tried in our lab on fruit juice and wines and it was found to be unsuitable because of a lack of sensitivity at low levels of acetate or in highly colored liquids. McCloskey (1975) found that most wines would have to be run undiluted or only slightly diluted in order to be within the capabilities of the above assay based on reactions 1 and 2. In red wines, this makes for optical densities that are so high so as to make the assay unfeasible on these wines. Postel and MacCagnan (1971) have used an enzymatic assay which utilizes the assay reactions in 1 and 2 above. They worked solely on white wines and were able to obtain reasonable analytical results. An assay for acetate was still sought in our lab which could easily be used on highly colored liquids, such as prune juice, Concord grape juice, and red wine.

Meyers et al. (1974), in an unpublished report, described an assay reaction that appeared suitable for the measurement of acetate in biological fluids where the level of acetate is on the order of 5–10 μg/ml. Their assay utilizes three coupled enzymatic reactions catalyzed, respectively (reactions 3–5), by acetate kinase (AK), pyruvate kinase

$$acetate + ATP \xrightleftharpoons{AK} acetyl\ phosphate + ADP \qquad (3)$$

$$ADP + phosphoenolpyruvate \xrightleftharpoons{PK} pyruvate + ATP \qquad (4)$$

$$pyruvate + NADH \xrightleftharpoons{LDH} lactate + NAD^+ \qquad (5)$$

$$acetate + phosphoenolpyruvate + NADH \rightleftharpoons lactate + acetyl\ phosphate + NAD^+ \qquad (6)$$

(PK), and lactate dehydrogenase (LDH). We attempted to use the procedure they described and found that the assay reaction proceeded at a slow rate (approximately 120 min for a reasonable optical density change) and that it was difficult if not impossible to determine when the assay reaction was over. Meyers et al. (1974) suggested that a timed assay be used, yet we found that acetate concentrations high enough to cause a reaction upset the equilibrium of the enzymatic reactions used so that reproducibility was difficult to obtain in a reasonable amount of time. The latter difficulties with the procedure were probably due to the reaction catalyzed by acetate kinase (from *Escherichia coli*) in reaction 3. This reaction is very unfavorable to the right (Rose, 1962) with a K_m for acetate of 0.30 M and a K_m of 0.005 M for acetyl phosphate. The apparent equilibrium constant at pH 7.3 was greatly in favor of phosphorylation of ADP, 0.006–0.11.

Because of the difficulties encountered with reactions 3–5 above, we decided to revamp the procedure in order to optimize the conditions for the assay reactions (3, 4, and 5). An attempt was made to drive the reactions to completion with help from the literature as to the optimum concentrations of ATP, Mg^{2+}, etc. (Boyer, 1962; Rose, 1955, 1962). Special attention was given to the K_m values for optimizing the assay reaction catalyzed by acetate kinase (Rose, 1962). The results of our work have yielded the enzymic assay described here, utilizing the reactions first described by Meyers et al. (1974), which is suitable for the

analysis of liquids such as fruit juices and their fermented products.

EXPERIMENTAL SECTION

Apparatus. The following equipment was used: a Bausch and Lomb (B&L) 70-6 spectrophotometer with a DR-37 digital display, B&L 1-cm (i.d.) round cuvets, various Eppendorff microliter type pipets and tips, Repipettes (Lab Industries), an Orion Model 407 specific ion meter (for pH), and Parafilm. A Cash type steam distiller was used for the distillation procedures.

Materials. The enzymatic assay requires a spectrophotometer capable of measuring the absorbance of nicotinamide adenine dinucleotide (reduced form, NADH) at 340 or 366 nm, 1-cm cells. The enzyme reagents include: buffer, coenzymes, cofactors, and three enzymes. For the distillation of the samples followed by detection by titration with a known base a steam distiller is needed.

Buffer Solution 1. The assay reactions were carried out at the following final concentrations as to this reagent: pH 7.4 ± 0.03 and 93 mM triethanolamine, 67 mM K^+, and 7 mM Mg^{2+}.

The reagent was made as follows. Dissolve 5.55 g of triethanolamine (BMC, Catalog No. 15325) in approximately 275 ml of distilled or deionized water along with 1.51 g of potassium chloride (KCl, MCB No. 7412) and 0.459 g of magnesium chloride ($MgCl_2 \cdot 6H_2O$, Mallinckrodt No. 1410). Adjust the pH to 7.46 with about 1.5 ml of 5 N KOH and bring to a volume of 300 ml. This buffer is stable for about 2 weeks at 0–4 °C, but the pH should be checked every 2 or 3 days.

Solution 2. This solution contains the coenzymes in a sodium bicarbonate buffer along with phosphoenolpyruvate (PEP). The following procedure was used. Dissolve 1.000 g of adenosine triphosphate ($ATP \cdot Na_2$, BMC Catalog No. 15028), 50 mg of NADH (BMC Catalog No. 15142), 100 mg of phosphoenolpyruvate (BMC Catalog No. 15296), and 750 mg of sodium bicarbonate ($NaHCO_3$, Mallinckrodt 7412) in 10.0 ml of distilled water. This reagent is not stable for over 7 days at 0–4 °C.

Enzyme Solution 3. The enzymes include pyruvate kinase and lactate dehydrogenase (respectively, PK and LDH, BMC Catalog No. 15528). Both of the enzymes are from rabbit muscle. A suspension of 4 mg/ml is necessary, where there is about 600 IU per ml of each of the enzymes. Ten microliters of this suspension contains 5.5 IU of lactate dehydrogenase and 6.0 IU of pyruvate kinase.

Enzyme Solution 4. Solution 4 consists of acetate kinase (AK, BMC Catalog No. 15089). A preparation of 5 mg/ml is necessary where 10 μl of the suspension contains 8.5 IU of enzyme.

Procedure. *Sample Preparation for the Enzymic Assay.* Samples are prepared only with a dilution step, unless samples contain protein or an excessively large amount of insoluble solids. The aim is to bring the expected acetate within a range of 1–20 mg/100 ml and to lessen the optical density of the sample sufficiently so that extremely high initial absorbance values (E_1) are not incurred. For white samples like apple juice or white grape juice products no dilution may be necessary. Those samples which are dark, such as prune juice, red grape juice, and red wines, will require some dilution to eliminate high absorbance due to sample color. Usually a 2–5 times dilution will suffice.

Assay. The assay is performed as follows. Add to a cuvet 3.00 ml of buffer solution 1, 0.100 ml of solution 2, and 0.010 ml of enzyme solution 3 along with 0.100 ml of the prepared sample. Read the initial absorbance (E_1) at 340 nm after 2–3 min. Next, add 0.010 ml of enzyme

Table I. Statistical Data on the Enzymatic Assay for Acetate[a]

	Apple juice	Wine	H₂O–acetate
No. of assays	10	9	10
Mean	21.51	33.23	10.79
Range	20.77–22.73	30.61–35.85	10.38–11.22
Coeff of variation	1.1%	5.1%	3.2%
SD	0.249	1.697	0.349
Dilution factor	0	2	0

[a] All the concentrations are in mg/100 ml.

solution 4 and incubate the mixed contents at 29 °C for 40–65 min, then read the final absorbance (E_2) and calculate the change in absorbance ($E_2 - E_1$).

Calculations. The concentration of acetate anion (or as acetic acid) is determined from a standard curve made by running various solutions of sodium acetate and water. The use of fresh reagents will enable the analyst to use the Beer–Lambert equation where the molar extinction coefficient of NADH is 6220 (Kaplan, 1962). An assay time of 60 min should be used for the latter calculation. A blank must be run and used in all calculations since there is a significant blank reaction when using the Boehringer Mannheim reagent, acetate kinase. This blank is usually 0.080 to 0.110 absorbance unit, where a water sample is run.

RESULTS AND DISCUSSION

The enzymatic assay presented herein offers significant advantages for the measurement and detection of acetate in food products such as juices and fermented beverages. The method requires very little if any sample preparation. Only a dilution step is necessary for some fermented beverages which may be high in acetic acid, e.g. over 20 mg/100 ml. Also, the procedure has most of the advantages of a good analytical technique, including: precision, good recovery, and specificity coupled with speed. The assay has been used for the past year in a commercial winery for the analysis of grape juice and red wines. It has proved far superior in all ways to the steam distillation procedure (Amerine, 1965) in common usage in that industry. The procedure has also been used in a private laboratory for assaying fruit juices. The assay may be very valuable as a common analytical technique because of its accuracy over the steam distillation methods and because it is a simpler technique than gas chromatography.

The statistical data gathered for the enzymatic assay are presented in Table I for apple juice, white wine, and an acetate–water mixture. The results were very good with a standard deviation as good as 0.25 mg/100 ml at 21 mg/100 ml for multiple determinations upon apple juice. Similar results were obtained for the acetate–water mixture. The white wine assayed with a standard deviation of 1.7 mg/100 ml after a two times dilution. In our hands the steam distillation procedure (Amerine, 1965) was not capable of yielding results below 20 mg/100 ml of acetic acid and had a standard deviation on the order of 10–20 mg/100 ml with disturbingly poor recovery of acetic acid (see below).

The distillation procedure (Amerine, 1965) was tested on a water–acetic acid solution made up to a concentration of 52.5 mg/100 ml. Analysis of multiple distillates by titration with a known base showed there was a 70% recovery of the added acetic acid while the analysis of the same distillate with the enzymic method showed there was a 71% recovery of the added acetic acid. Also, four wines were assayed with both procedures and the distillation

Table II. Recovery of Acetic Acid from Fruit Juices and Wines

Sample	Endog. HAc, mg/100 ml	Added HAc, mg/100 ml	% recovy
Apple juice	3.00	18.15	101.8
Prune juice	9.88	17.32	100.1
Concord grape juice	6.22	35.94	100.7
White wine	27.53	35.94	98.7
Red wine	35.94	35.94	96.9
HAc + water		11.13	97.0

Table III. Interference from Volatile Acids and Acetate Esters

Sample	mg/100 ml	Enzymatic HAc
Ethyl acetate	89.4	0.00
Propyl acetate	44.4	0.00
Butyric acid	87.6	0.20
Propionic acid	100	0.26

procedure only recovered an average of 83% of the enzymatic assay values for the four wines. The range for the latter analysis was 71–91%, indicating that the distillation procedure is not very reproducible from wine to wine. These results were confirmed by McCloskey (1975) in a study of the distillation procedures where 22 wines were tested. Of these wines the distillation procedure was 27–127% of the enzymic assay values. These types of results are commonly reported by those using steam distillation procedures to assay fruit juices and their fermented products. This indicates the need for a better procedure for the determination of acetic acid in these liquids. This is especially true for those products containing less than 20–30 mg/100 ml of acetic acid.

Results of the recovery of acetate with the enzymic assay are presented in Table II. The assay was capable of recovering essentially all of the acetate added to water, three fruit juices, and two wines. The assay recovered from 96.9% of the acetic acid in red wine, the most difficult sample to work with, to 101.8% of the acetic acid in apple juice. The recoveries with a prune juice sample and Concord grape juice were, respectively, 100.1 and 100.7%. The range of recovery with the enzymic assay was 97–102%.

The enzymic assay was tested for interference from acetate esters and other volatile acids. Ethyl acetate and *n*-propyl acetate were tested because they are common in fermented beverages such as wine (Daubt and Ough, 1973). The results are presented in Table III, along with data on the interference from volatile acids. Neither the acetate esters nor butyric and propionic acids interfered in the assay.

Sources of Errors. It is important that the analyst understand that there is a significant blank reaction and because of this Figure 1 is included. There seems to be some acetate or ADP contamination of the acetate kinase used (BMC) since the curve shows an initial substrate reaction, indicated by the initial rapid decrease in absorbance, due to the addition of the acetate kinase. This rapid decrease in absorbance is followed by a steady decline for up to 2 h at a rate of 0.020 absorbance unit per 15 or 20 min. These data indicate the absolute necessity of blank data for all calculations. In addition, it is also necessary to dispense equally and accurately the acetate kinase reagent. The latter steady decline in absorbance is probably due to an ADP generating system (e.g., ATPase) or to an NADH oxidase.
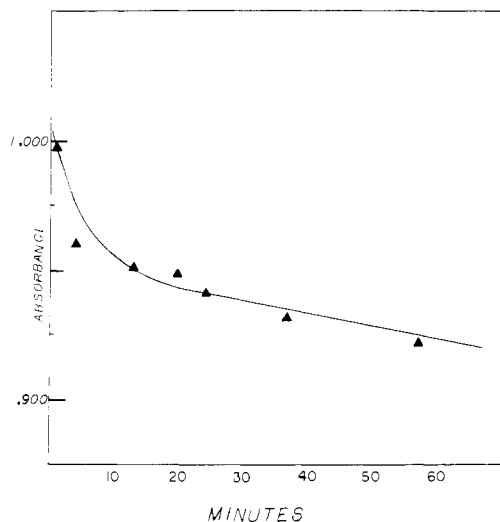
Figure 1. Blank reaction curve showing the nature of the reaction.

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Spectrofluorometric Determination of β-Asarone in Sweet and Dry Vermouths

Edward J. Wojtowicz

A spectrofluorometric method for the quantitative determination of β-asarone in sweet and dry vermouths has been developed. After steam distillation of the β-asarone from the vermouth, the compound is partitioned into hexane, which is then washed with 1 N NaOH, 1 N HCl, and water. The β-asarone is then determined fluorometrically. The presence of β-asarone is confirmed by thin-layer chromatography of the same solution (0.5 μg detectability). The procedure gives greater than 90% recovery of 1 ppm of β-asarone in both sweet and dry vermouths.

Oil of calamus, derived from the dried rhizome of *Acorus calamus* Linn., is used as a minor flavoring agent at levels below 5 ppm and up to 10–30 ppm in bitters, liqueurs, and vermouths (Miller, 1973). Studies (Gross et al., 1967; Taylor et al., 1967) have shown that malignant intestinal tumors form in rats fed a diet containing up to 5000 ppm of oil of calamus. Use of the root, oil, or extract in food has been banned in the United States (Code of Federal Regulations, 1974a); however, it is still used in certain European countries (Hall, 1973).

The β-asarone content of the oil derived from the tetraploid variety of Indian *Acorus calamus* Linn. has been reported to be 82% (Guenther, 1952) while oil from the European variety contains about 5% β-asarone (Larry, 1973).

Column chromatography (Chopra et al., 1965) and gas–liquid chromatography (Larry, 1973; Usseglio-Tomasset, 1966) have been used for the quantitative determination of β-asarone, while thin-layer chromatog-

raphy (TLC) (Stahl, 1965) has been used for the qualitative identification of the components in calamus.

These methods lack sensitivity in the fractional part per million range which is required when dealing with a carcinogenic compound. β-Asarone is the cis isomer of 2,4,5-trimethoxy-1-propenylbenzene, and lends itself well to fluorescence analysis. A search of the literature showed no previous reference to the use of fluorescence for β-asarone analysis.
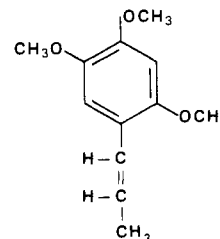
A method has been developed that utilizes spectrofluorometry for quantitative estimation of β-asarone in sweet and in dry vermouths and TLC for qualitative identification.

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