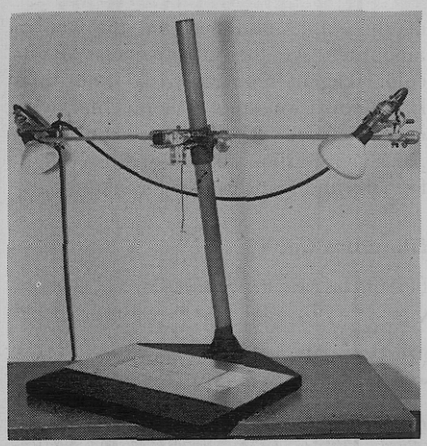

Figure 1. Horizontal easel, with stainless steel sheet, supports camera and lights

sults have been obtained on papers exposed during stain development to air having relative humidities in the range 21 to 40% of saturation at about 25° C. In contrast excessive and rapid darkening has occurred when relative humidities of about 15% or lower were encountered. For this reason humidification may be necessary for satisfactory staining in arid localities.

For viewing finished color slides, use a 300-watt projector fitted with a 3-inch lens which permits table top projection at very short lens-to-screen distances with brilliant color reproduction.

The final colors obtained with some known substances are given in Table I. Examples of stained chromatograms of sapogenins and saponins from alfalfa are shown in Figure 2. The smallest

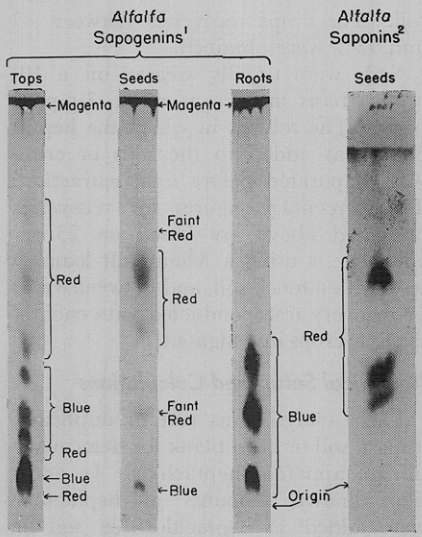

Figure 2. Ascending paper chromatograms stained with sulfuric acid to acetic anhydride 1 to 1 by volume

1. Developing solvent. Ethyl acetate–95% ethyl alcohol–0.05N ammonium hydroxide, 68:15:13.5
2. Developing solvent. *n*-Butyl alcohol–acetic acid–water, 65:10:32 (upper phase)

quantities of single triterpenoids detected were 5 to 10 γ. Somewhat larger quantities produce very intense stains.

Table I. Triterpenoids and Steroids Stained with Liebermann-Burchard Reagent

Test Substance	*Final Color*
Abietic acid	Red
Castanogenin	Blue
Cholesterol	Purple
Cholic acid	Red
Echinocystic acid	Red
Erythrodiol	Red
Hederagenin	Blue
Medicagenic acid	Blue
Oleanolic acid	Magenta
Sitosterol	Purple
Smilagenin	Purple
Soyasapogenol B	Red
Soyasapogenol C	Magenta
Ursolic acid	Red
Uvaol	Magenta

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MUSHROOM ANALYSIS

Chromatographic Identification of the Amino Acids and Carbohydrates in the Cultivated Mushroom *Agaricus Campestris* L. exFries

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In recent years the cultivated mushroom, *Agaricus campestris* L. exFries, has become prominent in the American diet. Correspondingly the mushroom industry has become a more important member of our agricultural community. The increased popularity is due to the high nutritive value and low caloric content of the cultivated mushroom. These properties were recognized and partially measured by use of the microbiological assay and standard chemical methods by a group of workers at the University of Massachusetts around 1940. Their work has been summarized by Esselen and Fellers (*5*). With the advent of chromatographic techniques (*1, 8*), it has become possible to identify the amino acid and carbohydrate spectra of the mushroom. A number of amino acids and carbohydrate constituents previously unrecognized in the cultivated mushroom are reported herein.

Composition studies utilizing microbiological assay, as an analytical tool, were conducted by Esselen and Fellers (*5*) in 1946. These workers identified and measured quantitatively six of the amino acids known to be essential to man. The remaining four essential amino acids were identified by qualitative chemical tests. In 1951, Block, Stearns, Stephens, and McCandless (*2*), using two-dimensional chromatography, found 12 amino acids present in the sporophore of *Agaricus campestris*. These studies represent the present status of knowledge, in this country, of the amino acid content of the cultivated mushroom. In 1953, two-dimensional chromatography employed on a closely related species, *Agaricus hortensis* var. *alba*, identified 16 and possibly 21 amino acids in their alcohol extracts and acid hydrolyzates (*16*).

The carbohydrate moiety of *Agaricus campestris* was investigated by McConnell and Esselen (*11*) in 1947. Employing standard chemical methods they identified and quantitatively measured: mannitol, reducing sugars in the form of dextrose, glycogen, crude hemicellulose, and furfural-yielding substances. The above study, as far as it is known, is the only report in the literature on the carbo-

The amino acid and carbohydrate composition of the cultivated mushroom, *Agaricus campestris*, was investigated using one- and two-dimensional chromatography. Twenty-seven ninhydrin reacting substances, including eight essential amino acids, as well as 17 carbohydrates were present. A polyhydric alcohol, mannitol, was present in relatively large quantity. A modification of the method of Bradfield and Flood, in which polyhydric alcohols fluoresce under ultraviolet light, is suggested as a sensitive method for detecting these compounds. The hexoses, glucose and galactose, were present in amounts comparable to mannitol. The intensity of spots on paper chromatograms demonstrated that the tyrosine content progressively decreased through the first, third, and fifth crops. The ease of enzymatic discoloration correlates with the levels of tyrosine in the three crops.

hydrate materials present in the cultivated mushroom.

Materials and Methods

The mushrooms used in these analyses were obtained from several commercial growers in Delaware. Fresh, tight-veiled mushrooms, with a cap diameter of approximately $1^1/_2$ inch were selected and dried in a forced-draft oven at 80° C. for 8 hours. The dried material was ground in a Wiley mill to pass a 40-mesh sieve. The dried, ground samples were stored in bottles placed in desiccators. The average water content of the fresh mushrooms was 91.8%.

Amino Acids. One-gram samples of the dried, ground material were extracted with three successive 25-ml. portions of boiling 80% ethyl alcohol. In each case the tubes containing the mushroom sample and hot alcohol were extracted for 1 hour on a shaker. The tubes were centrifuged and the alcohol portion was decanted. The combined alcohol extracts were evaporated under reduced pressure in a 50° to 55° C. water bath to a volume of approximately 1 ml. The concentrate was diluted to 3 ml. with 50% isopropyl alcohol. To remove interfering salts, the resulting solution was placed in a freezer overnight and filtered.

The residue from the centrifuged portions was suspended in 40 ml. of 6*N* sulfuric acid and hydrolyzed for 24 hours on a steam bath. The hydrolyzate was adjusted to pH 6.5 to 6.8 with calcium carbonate suspension and the white precipitate removed by centrifugation. The liquid was decanted, evaporated to 1 ml. and diluted to 3 ml. with 50% isopropyl alcohol, and the interfering salts were removed by freezing.

Aliquots of the alcohol extract and acid hydrolyzate were subjected to one-dimensional ascending paper partition chromatography, using four-step irrigation for maximum resolution. This modification of a standard method, developed by Lynch (*4*), was employed for its ease of operation and its excellent resolving power. Butanol–acetic acid–water in a ratio of 4–1–5 was used as the resolving solution followed by a 2% ninhydrin in *n*-butyl alcohol spray to detect the amino acids. The identification of the various ninhydrin-reacting compounds was accomplished by running aliquots of known amino acids alongside the mushroom samples on Whatman No. 1 filter paper sheets. Two-dimensional chromatograms, following the method of Levy and Chung (*9*), were made. These not only served to confirm the results of the one-dimensional system employed, but they also completely separated the remaining few close-running amino acids. Confirming results were obtained for amino acids present in low titer with standard qualitative chemical tests. The Hopkins-Cole test and the Acree-Rosenheim formaldehyde reaction were used to detect tryptophan. Alkaline lead acetate was used to identify cystine. The Sakaguchi test was employed for arginine, and Knoop's test for histidine. Citrulline gives a yellow color when a fresh chromatogram is sprayed with a 1% solution of *p*-dimethylaminobenzaldehyde.

Carbohydrate Constituents. The alcohol extracts and acid hydrolyzates were obtained by the procedure described above, except that 2*N* sulfuric acid was used for the hydrolysis of the samples. The neutralized supernatant liquid was evaporated to dryness and the resulting residue treated with three successive 10-ml. portions of dry pyridine to extract the sugars. The pyridine-sugar solution was filtered, the pyridine was removed under diminished pressure, and the dried sugars were dissolved in 2 ml. of 50% isopropyl alcohol. This solution was used for the determinations.

The reducing sugars were identified using the method of Partridge (*13–15*). The presence of sugar alcohols was determined by the method of Bradfield and Flood (*3*). The amino sugars were determined using the Gray (*6*) solvent and the Morgan and Elson (*12*) indicator. The general procedure is outlined by Lynch, Wright, and Olney (*10*).

Results and Discussion

Amino Acids. Twenty-seven ninhydrin reacting compounds were separated and 23 of these have been identified as amino acids or amino acid derivatives. Qualitatively, the free amino acids identified in the alcohol extract are similar to the protein amino acids identified in the hydrolyzate. Glutamine, asparagine, and tryptophan are present in the alcohol extract, but absent from the hydrolyzate. Under the hydrolytic conditions employed glutamine and asparagine are deamidated and tryptophan is destroyed. Three of the unidentified compounds have R_f values greater than leucine, while the remaining unknown has an R_f intermediate between threonine and α-alanine. Two of the fast running unknowns appeared in good titer in the alcohol extract. All three were obtained upon hydrolysis. These unknowns with high R_f values can be preferentially extracted with cold 80% aqueous solutions of ethyl or isopropyl alcohols.

A comparison of the results obtained with those of Block *et al.* (*2*), Esselen and Fellers (*5*), and the related species of Renard and Casimir (*16*) is shown in Table I. This paper reports 10 additional amino acids: asparagine, glutamine, β-alanine, serine, isoleucine, methionine, cystine, citrulline, γ-aminobutyric acid, and proline.

Of the eight amino acids known to be essential to man, all are present, in varying amounts, in *Agaricus campestris*. In order of decreasing concentration they appear to be: lysine, threonine, valine, isoleucine, leucine, methionine, phenylalanine, and tryptophan. Of the many amino acids present, those that appear to be in the highest concentration are: α-alanine, glutamic acid, serine, glycine, proline, lysine, threonine, valine, aspartic acid, isoleucine, leucine, and tyrosine.

One of the perplexing problems of mushroom culture is that the first crop of mushrooms is more sensitive to enzymatic browning and injury by bruising than the succeeding crops. (A mushroom bed produces a series of seven crops, on the average, over a time span of 8 to 10 weeks.) The discoloration is presumably due to the action of tyrosinase on tyrosine. This problem is important as approximately one third of the total production is harvested with the first crop.

The first step taken in attempting to understand this difference was a Kjeldahl nitrogen comparison of the first, third,

Table I. Amino Acids Identified by Various Workers in Two Related Species of Cultivated Mushrooms

Amino acid	Esselen and Fellers[a]	Block et al.[a]	Renard and Casimir[b]	Present Paper[a]
Lysine	X[c]	X	X	X
Histidine	X[c]	X	X[d]	X
Arginine	X	X	X[d]	X
Threonine	X[c]	X	X	X
Tryptophan	X	X		X
Phenylalanine	X[c]	X	X[d]	X
Leucine	X	X[d]	X[d]	X
Isoleucine	X	X[d]		X
Methionine	X	X[d]	X[d]	X
Valine	X	X	X[d]	X
Tyrosine		X	X	X
α-Alanine		X	X	X
Glutamic acid		X	X	X
Aspartic acid		X	X	X
Glycine		X	X[d]	X
Serine			X[d]	X
Proline			X	X
γ-Amino butyric acid			X	X
Cysteic acid			X	
Cystine			X	X
Ornithine			X	
Citrulline			X	X
Asparagine				X
Glutamine				X
β-Alanine				X
			3 spots unidentified	4 spots unidentified

[a] Analyses made with *Agaricus campestris* L. exFries.
[b] Analyses made with *Agaricus hortensis* var. *alba*.
[c] Identified only by qualitative chemical tests.
[d] Not separated by technique used, presence of only one is assured.

Table III. Sugars and Related Carbohydrates Present in Acid Hydrolyzates of the Cultivated Mushroom, *Agaricus Campestris*, as Determined by Chromatographic Analyses

I.	Reducing sugars	
	A. Pentoses	
	Xylose	++
	Ribose	+
	B. Methylpentoses	
	Rhamnose	++
	Fucose	+
	C. Hexoses	
	Glucose	+++
	Galactose	+++
	Mannose	++
II.	Amino sugars	
	Glucosamine	+
	N-Acetylglucosamine	+
III.	Sugar alcohols	
	Mannitol	+++
	Inositol	+
IV.	Sugar acids	
	Galacturonic acid	++
	Glucuronic acid	+
	One unknown	+
V.	Methylated sugars	
	Two unknowns	+
VI.	Methylated sugar acids	
	One unknown	++

+ Indicates relative amount.

and fifth crops. As shown in Table II, no significant difference between crops appears, and no trend in total nitrogen content is evident.

Some light was shed on this problem when carefully prepared alcohol extracts of the first, third, and fifth crops were chromatographed. Of the 25 spots which appeared, only one amino acid, tyrosine, differed in intensity between crops (Figure 1). The tyrosine spot was strong in the first crop, weak in the third, and barely visible in the fifth. This is consistent with the hypothesis that the browning results from the action of tyrosinase on tyrosine.

Carbohydrate Constituents. In the qualitative analyses of the acid hydrolyzates (Table III), two pentoses (xylose and ribose), two methylpentoses (rhamnose and fucose), three hexoses (glucose, galactose, and mannose), two amino sugars (glucosamine and *N*-acetylglucosamine), two sugar alcohols (mannitol and inositol), two sugar acids (galacturonic and glucuronic acid), a uronide of low R_f value that probably was mannuronic acid, and two unidentified methylated sugars were found. Present in good titer was a compound with an R_f value and reddish color corresponding to a methylated uronide (7).

Table II. Total Nitrogen Content of Successive Mushroom Crops from Three Delaware Growers

	Total Nitrogen, %			
	Grower			
Crop	A	B	C	Average
First	5.19	4.36	4.58	4.71
Third	4.78	4.31	4.36	4.48
Fifth	5.83	4.72	4.23	4.93

All determinations were run in triplicate.

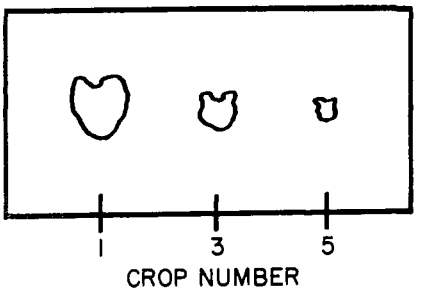

Figure 1. The tyrosine difference of successive crops

Reproductions of tyrosine spots on one-dimensional chromatograms of the ethyl alcohol extracts of various crops of fresh mushrooms

The qualitative analyses of the alcohol extracts demonstrated the presence, in very low titer, of glucose, galactose, and mannose. After development of the chromatograms employed to detect polyhydric alcohols (3), it was noted that the mannitol and inositol spots fluoresced strongly when placed under ultraviolet light. They appeared as salmon-colored, fluorescent spots against a blue background. This appears to be a very sensitive method for the detection of these compounds.

The carbohydrate constituents that appear to be in the greatest quantities are galactose, mannitol, and glucose, followed by rhamnose, xylose, mannose, galacturonic acid, and the unidentified methylated uronide.

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Effect of Fat on Carotene Stability in Dehydrated Alfalfa

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The influence of various fats on carotene retention in different samples of dehydrated alfalfa was investigated. Some samples of dehydrated alfalfa with added fat showed greatly improved carotene retention, while others did not. Carotene retention was not improved by antioxidants at the level normally used for the stabilization of fats; however, the use of antioxidants at higher levels may improve carotene retention. Fatty acids did not increase carotene destruction in dehydrated alfalfa, but caused color deterioration.

THE importance of dehydrated alfalfa as a source of carotene in feeds has made it imperative that studies be conducted on the stability of this provitamin. Research conduted by Guilbert (*7*), Fraps and Treichler (*6*), Fraps and Kemmerer (*5*), Taylor and Russell (*16*), Wilder and Bethke (*18*), Mills and Hart (*10*), Halverson and Hart (*8*), and many others has shown that the carotene content of dehydrated alfalfa decreased during storage. The rate of decrease was influenced mainly by oxygen and storage temperatures.

With the recent use of increasing amounts of fat in the alfalfa dehydrating industry, it became important to study the effect of fats and antioxidants on carotene stability in dehydrated alfalfa during storage. Mitchell, Beauchene, and Silker (*11*, *13*) showed that oil added to dehydrated alfalfa at the rate of 80 pounds per ton produced greater carotene stability than when added at the rate of 16 pounds per ton. They (*13*) showed that heating the meal for 1 hour at 100° C. after spraying with oil gave a further increase in carotene stability. Increasing the amount of fat from 1 to 5% in dehydrated alfalfa was found by Bickoff *et al.* (*3*) to improve carotene retention. Studies by Livingston, Bickoff, and Thompson (*9*) revealed that the addition of animal fats in combination with an antioxidant, 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline (Santoquin), improved the retention of carotenoids in mixed feeds more effectively than when the oil and antioxidant were added separately. Siedler, Enzer, and Schweigert (*15*) showed an improvement in carotene stability in mixed feeds by the use of stabilized fats. Thompson (*17*) reported that on increasing the amounts of antioxidants in alfalfa, carotene stability increased, but the level of antioxidant approached a limit (approximately 0.125%) above which no additional benefit was noted. It has also been reported that animal fats produced greater carotene stability in dehydrated alfalfa during storage than vegetable oils (*13*, *14*).

This study was designed to obtain more information concerning the effects of the addition of animal fats to samples of dehydrated alfalfa. The study encompassed the effects of stabilization of the animal fats with antioxidants, the effect of free fatty acids, and the effect of wide variations in melting point of the fats on the carotene stability in dehydrated alfalfa.

Methods

The melted fats at 80° C. were added dropwise to 200 grams of dehydrated alfalfa while it was vigorously stirred mechanically in a 3-necked distillation flask, placed in a heated water bath. Approximately 5 minutes were required to heat the alfalfa to a temperature of 80° C. and to add the fat. The alfalfa was then stirred at 80° C. for 10 minutes after the addition of the fat. Solvents were not used to introduce the fats, because of the possibility that they may bring the carotene and natural antioxidants into mutual solution to a greater extent than the fat alone, thereby improving carotene stability. Because heat is necessary for good fat dispersion and may improve carotene retention in normal pelleting operations (*13*), the above method of addition was used. Immediately after the mixing was completed, the samples were removed from the flask, spread out on paper to cool, and then placed in jars with screw-type lids. Control samples were treated in a similar manner without the addition of fat. The per cent retention of carotene, unless otherwise stated, was based on the carotene content of the control sample immediately after mixing. All samples were stored at 37° C.

Carotene determinations were made by using the AOAC method of analysis (*1*). Beauchene *et al.* (*2*) and Mitchell and Silker (*12*) have shown that the AOAC method of analysis gave high values for carotene in the presence of DPPD (diphenyl-*p*-phenylenediamine). In the following experiments, when DPPD was added to alfalfa at the rate of 0.0015% there was no color interference. Initially moisture determinations were made, but this was later discontinued, because of the slight variation which occurred at the beginning and after various lengths of time in storage. The alfalfa samples were obtained from commercial dehydrators located in various states. The time of the year when the samples were dehydrated was not known, but none of the samples had been pelletized or had added fat or antioxidant.

Results

The first experiment was designed to show the effects on the carotene retention in different samples of dehydrated alfalfa, of adding 3% bleachable fancy and No. 2 tallows stabilized with 0.05% of various antioxidants. Bleachable