Table I. Reflectance Measurements Using Hunter Color Difference Meter

Juice sample	% SS of tomato base	L (lightness)	a (redness)	b (yellowness)	a/b (hue)	$\sqrt{a^2 + b^2}$ (saturation)
I	5	27.50	24.82	13.78	1.801	28.40
II	10	27.10	24.10	13.64	1.765	27.10
III	16	27.01	23.45	13.51	1.736	27.09
IV	20	26.86	22.77	13.49	1.689	26.53

Table II. Determination of Pigment Concentration

Juice sample	Total carotenoids,[a,b] mg/g of juice	Lycopene,[a] mg/g of juice
I	0.050	0.037
II	0.044	0.033
III	0.040	0.029
IV	0.032	0.016

[a] Calculated from absorbance at 470 nm. [b] Expressed as lycopene.

in hexane by Zechmeister (1962). The decrease in lycopene content reported here was due to an actual degradation of lycopene, rather than to a progressive conversion from the *all-trans*-lycopene to a less strongly colored, less intensely absorbing cis form. This was shown by the similar shape of log absorbance *vs.* wavelength curves for the lycopenes isolated from the control and reconstituted juices. As described by Zechmeister (1962) an increase in absorbance at 360 nm, producing dissimilar log absorbance curves, would have indicated trans–cis isomerization of lycopene.

The decrease in total pigment or isolated lycopene correlated with the decrease in redness (as measured by Hunter "a/b") at the 1% level of significance (r = 0.978 and r = 0.907, respectively). This high correlation, with our preliminary evidence that in the sera browning (expressed as concentration of hydroxymethylfurfural) did not significantly occur upon paste concentration, suggests that the change in color, particularly in redness, of the juice products reconstituted from paste is a result of the degradation of the carotenoid pigments during the heat concentration of the paste. However, further investigation is needed to explain the darkening of the samples (as measured by decrease in "L") which occurs upon heat treatment.

ACKNOWLEDGMENT

The author thanks Christopher Gaul and Kathey Cunningham for technical assistance in this project.

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Received for review July 15, 1974. Accepted October 11, 1974. This project was funded by a grant from E. D. Smith & Sons, Ltd., Winona, Ontario.

Isolation and Identification of Acidic Oligopeptides Occurring in a Flavor Potentiating Fraction from a Fish Protein Hydrolysate

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An enzymatic hydrolysate of a fish protein concentrate was fractionated to obtain a low molecular acidic fraction having a flavor potentiating activity. Almost 30 acidic oligopeptides were isolated from this fraction. By chemical and mass spectrometric methods, the complete or partial amino acid sequences of these oligopeptides were determined as follows: dipeptides, Ala-Glu, Asp-Ala, Asp-Gly, Asp-Leu, Glu-Asp, Glu-Glu, Glu-Gly, Glu-Ser, Ile-Asp, Ile-Glu, Ser-Asp, Thr-Glu, Val-Asp, and Val-Glu; tripeptides, Asp-Glu-Ser, Glu-Asp-Glu, Glu-Asp-Val, Glu-Gly-Ala, Glu-Gly-Ser, Glu-Gln-Glu, (Glu,Ile)-Asp, Ile-Glu-Glu, and Ser-Glu-Glu; tetrapeptides, (Asp,Glu,Gly)-Asp, (Asp,Glu,Ser)-Asp, (Asp,Gly,Ser)-Glu, and (Glu,Ile, Leu)-Glu; pentapeptides, (Asp,Glu,Gly, Ser)-Asp, (Asp,Glu,Gly,Ser)-Glu, and (Asp,Glu,Ser,Thr)-Glu; hexapeptide, (Asp,Glu_2, Gly,Ser)-Thr. Among these, peptides consisting of high molar ratios of glutamic acid residue were found to have a flavor activity qualitatively resembling that of monosodium glutamate.

Although most enzymatic hydrolysates of food proteins generally have a flavor potentiating activity like that of monosodium glutamate (MSG), these are often accompanied by an unfavorable bitter flavor at the same time; factors responsible for the bitterness have been disclosed (Fujimaki *et al.*, 1968; Matoba *et al.*, 1969; Kirimura *et al.*, 1969; Arai *et al.*, 1970). Recently, several reports have been presented on peptides having a MSG-like flavor activity; these are hydrophilic glutamyl oligopeptides (Arai *et al.*, 1972, 1973). In our preceding paper we have reported that a fish protein concentrate (FPC) treated with

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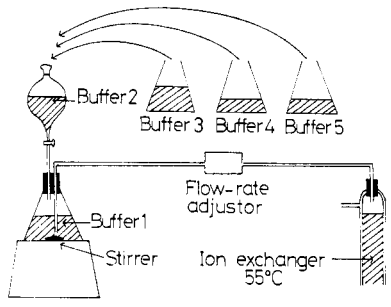

Figure 1. Method for separating acidic oligopeptides by Amberlite CG-120. The ion-exchanger column was equilibrated with buffer 1 (0.2 *M* pyridine acidified with acetic acid to pH 3.0) and maintained at 55°. A conical flask containing 800 ml of buffer 1 was set on a magnetic stirrer, and a separating funnel containing 800 ml of buffer 2 (0.3 *M* pyridine, pH 4.0) was connected with this flask. After a sample was applied on the column, the cock between buffer 1 and buffer 2 was opened, and the mixture sent there through a flow-rate adjustor (120 ml/hr). After buffer 2 was emptied out of the separating funnel, 800 ml of buffer 3 (0.4 *M* pyridine, pH 4.5) was added to the funnel. In a similar manner, 400 ml of buffer 4 (0.6 *M* pyridine, pH 5.0) and 400 ml of buffer 5 (2.0 *M* pyridine, pH 5.5) were used in this order. Every 10 ml of the eluent from the column was collected.

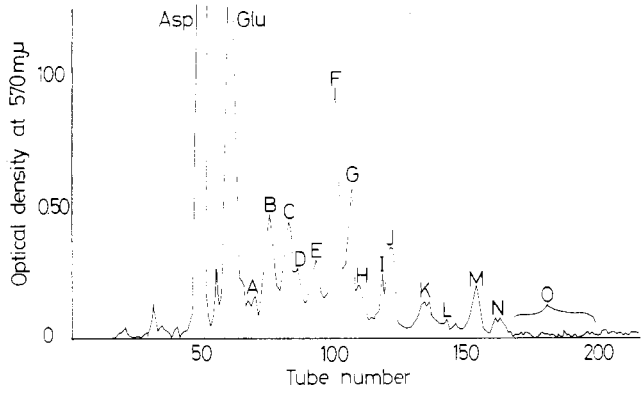

Figure 2. A chart showing free amino acids (aspartic and glutamic acids) and acidic oligopeptide fractions (A–O) separated by ion-exchange chromatography. No significant peaks were detected after tube number 200.

Pronase has a potent MSG-like flavor as well as a bitter flavor, and that a low molecular acidic peptide fraction contributes significantly to this MSG-like flavor activity (Fujimaki *et al.*, 1973).

The present paper deals with isolation and identification of acidic oligopeptides in the flavor fraction of an enzymatically hydrolyzed FPC.

MATERIALS AND METHODS

Substrate. An FPC preparation, EFP-90 (Nabisco-Astra Nutrition Development Corp., Sweden; $N \times 6.25 = 91.3\%$), was used as raw material. Nucleic acid related substances were removed therefrom by the method of Schneider (1946). The resulting tasteless FPC was used as a substrate for the following hydrolysis.

Enzymatic Hydrolysis. The substrate (100 g) was hydrolyzed with Pronase (Kaken Kagaku Co., Japan) under the following conditions: substrate concentration, 5%; enzyme–substrate ratio, 1/100; pH 7.0; temperature, 37°; and time, 96 hr. A small amount of toluene was added beforehand to the incubation system to prevent the microflora activity. The hydrolysate was heated at 100° for 15 min to inactivate the protease and centrifuged at 3000 rpm for 15 min. The resulting supernatant was treated twice with 4 l. of ether to remove toluene.

Ultrafiltration. The toluene removed supernatant was divided into four fractions of molecular weight higher than 10,000, 5000–10,000, and 1000–5000, and lower than 1000, by successive treatment with ultrafiltration membranes G-10T, G-05T, and G-01T (Nihon Shinku Co., Japan). As in the preceding report (Fujimaki *et al.*, 1973), a panel test has confirmed that the lowest molecular weight fraction has the most potent flavor activity. This fraction was lyophilized to obtain a powdered product with a yield of about 60 g from 100 g of substrate.

Fractionation. The lyophilisate (10 g) was chromatographed systematically on columns (each 3 × 60 cm) containing active carbon, Amberlite IRC-50 (pH 6.0), and Amberlite IR-45 (pH 3.5) by the method of Kroner *et al.* (1955), and divided into aromatic, basic, acidic, and neutral fractions with yields of 1.31, 1.81, 1.03, and 6.11 g, respectively. A panel test has also confirmed that the acidic fraction has the most potent flavor activity (Fujimaki *et al.*, 1973).

Separation of Acidic Oligopeptides. The low molecular, acidic fraction (400 mg on a dry matter basis) was chromatographed on a column (1.8 × 150 cm) containing Amberlite CG-120 (200–400 mesh, pH 3.0) by means of the gradient elution with the pyridine–acetic acid buffer system shown in Figure 1. Every 10 ml of the eluent was collected and its ninhydrin response determined by the usual method (Yemm and Cocking, 1955). These chromatographic conditions were effective in removing free aspartic and glutamic acids from peptides and also in separat-

Table I. Subfractionation of Fractions A–O[a] by Paper Partition Chromatography;[b] Numbers of the Subfractions and Their R_f Values

Fraction	Subfraction no.	R_f (×100)	Fraction	Subfraction no.	R_f (×100)
A	1	48	G	*4*	45
	2	29		*5*	32
	3	27		6	26
B	1	53	H	*1*	61
	2	42		*2*	57
	3	36		3	37
	4	34		4	32
	5	29		*5*	27
C	1	41	I	1	70
	2	33		*2*	59
	3	29		3	46
	4	24		4	17
D	*1*	37	J	*1*	58
	2	35		2	41
	3	31		*3*	33
	4	26	K	*1*	65
E	1	72		2	55
	2	58		*3*	26
	3	41		4	21
	4	34	L	*1*	65
	5	32		*2*	30
	6	30		3	25
	7	23	M	*1*	32
F	*1*	62		2	22
	2	43	N	*1*	69
	3	38		2	31
	4	35		3	26
	5	33	O	1	46
G	1	70		*2*	43
	2	56		3	34
	3	48		4	17

[a] See Figure 2. [b] The subfractions shown in italics were subjected to the following treatment.

Table II. Subfractionation of Fractions A-2-O-2[a] by Paper Electrophoresis;[b] Numbers of the Subfractions and Their Migration Distances

Fraction	Subfraction no.	Migration distance, cm	Fraction	Subfraction no.	Migration distance, cm
A-2	1	4.2	F-5	1	4.0
	2	3.4		*2*	2.8
A-3	1	4.8	G-4	1	3.5
	2	3.7		*2*	2.9
	3	3.5	G-5	*1*	3.4
B-3	*1*	4.9	H-1	1	3.1
B-4	*1*	5.3		*2*	2.4
	2	3.9	H-2	*1*	2.6
	3	2.2	H-5	*1*	3.1
C-2	*1*	4.0		2	2.3
C-3	1	4.5	I-2	*1*	3.0
	2	3.7	J-1	*1*	2.7
D-1	*1*	3.5		2	2.0
	2	2.8	J-3	1	4.9
E-4	1	3.9		*2*	3.8
	2	3.2	K-1	*1*	5.2
E-5	1	3.6		2	3.5
	2	2.3	K-3	1	3.3
E-6	*1*	3.1		*2*	2.7
F-1	1	4.8	L-1	1	5.0
	2	3.2		*2*	2.1
F-2	1	3.9	L-2	*1*	3.1
	2	3.1	M-1	*1*	3.2
F-3	*1*	4.9	N-1	1	3.8
	2	3.4		*2*	2.6
F-4	*1*	5.1	O-2	1	3.1
	2	4.1		*2*	2.2
	3	1.8			

[a] See Table I. [b] The subfractions shown in italics were subjected to the following treatment.

Table III. Subfractionation of Fractions A-2-2-O-2-2[a] by Thin-Layer Chromatography;[b] Numbers of the Subfractions and Their R_f Values

Fraction	Subfraction no.	R_f (×100)	Fraction	Subfraction no.	R_f (×100)
A-2-2	*1*	29	G-4-2	*1*	44
A-3-3	*1*	35	G-5-1	*1*	38
B-3-1	*1*	39	H-1-2	*1*	63
B-4-1	*1*	36	H-2-1	*1*	58
B-4-2	*1*	40	H-5-1	*1*	31
	2	32		2	20
C-2-1	*1*	35	I-2-1	*1*	62
C-3-2	*1*	30	J-1-1	*1*	56
D-1-1	*1*	36	J-3-2	*1*	32
E-4-2	*1*	37	K-1-1	*1*	65
	2	31	K-3-2	1	67
E-5-2	1	43		*2*	29
	2	35		3	23
E-6-1	*1*	34	L-1-2	*1*	67
	2	27	L-2-1	*1*	33
F-1-2	*1*	61		2	26
F-2-2	*1*	43	M-1-1	*1*	30
F-3-1	*1*	42	N-1-2	*1*	69
F-4-1	1	49		2	33
	2	39	O-2-2	*1*	45
	3	27			
F-5-2	1	53			
	2	42			
	3	35			

[a] See Table II. [b] The subfractions shown in italics were used as samples for the structural analyses.

ing the peptides into major peak components (Figure 2).

Paper Partition Chromatography (Ppc). To isolate peptides as the peak constituents described above, ppc was employed on a preparative scale as follows: paper, Toyo filter paper No. 50 (40 × 40 cm); sample size for one run, 5 mg (on a dry matter basis); solvent, 1-butanol–acetic acid–water (4:1:2); and operation temperature, 20°. The degree of separation by ppc was checked by treating a paper strip with ninhydrin. The separated zones were respectively extracted with sufficient amounts of water at 70° and each extract was concentrated *in vacuo*.

Paper Electrophoresis (PEP). Each concentrate was subjected to the following paper electrophoresis: paper, Toyo filter paper No. 51 (12.5 × 26 cm); sample size for one run, 2 mg (on a dry matter basis); buffer, pyridine–acetic acid–water (10:0.4:90, pH 6.5); voltage, 30 V/cm; and time, 1 hr. Zones were separated mostly with movement toward the anode and each separated zone was extracted as mentioned above.

Thin-Layer Chromatography (Tlc). A 20 cm long glass plate coated 0.5 mm thick with silica gel G (Merck) was used. Development was made with 1-butanol–acetic acid–water (4:1:2) at 20° for about 3 hr. The separated zones were extracted similarly.

Amino Acid Analysis. A sample (1 mg) was hydrolyzed with 6 *N* HCl (1 ml) in a deaerated test tube at 110° for 20 hr and the hydrolysate subjected to a Hitachi amino acid analyzer (KLA-5A).

Mass Spectrometry. A sample (0.2 μmol) was converted to its *N*-acetyl permethyl derivative by the method of Morris and Williams (1971) and analyzed with a Hitachi mass spectrometer (RMU-6L) under the following conditions: ionization voltage, 70 eV; temperature, 150°; and vapor pressure, 0.2×10^{-6} mm.

N- and C-Terminal Amino Acid Determination. The dansylation (DNS) method of Gray and Hartley (1963) and the revised hydrazinolysis method of Braun and Schroeder (1967) were applied to determine the N- and C-terminal amino acid residues, respectively.

RESULTS AND DISCUSSION

When the low molecular acidic fraction from the FPC hydrolysate was chromatographed on Amberlite CG-120, the 15 main peaks A–O were detected with clear separation from free aspartic and glutamic acids (Figure 2). The ppc treatment on each of these peak fractions gave almost 60 main subfractions as shown in Table I. Each subfraction was further separated as in Table II, when treated by paper electrophoresis. Similarly, when each of the paper electrophoresis subfractions was treated by tlc, almost 50 components were obtained as chromatographically homogeneous peptides (Table III). The analytical studies with 31 of these homogeneous peptides gave results on their amino acid compositions, N- and C-terminal amino acid residues, mass fragments, and identities concluded based on these data (Table IV). Figure 3 shows a representative mass spectrum taken of the acetyl permethyl derivative of Glu-Gly-Ala (fraction O-2-2-1). There is a parent peak (M^+) at *m/e* 387; this result rules out the possibility of any multiple structures of Glu-Gly-Ala. The major peaks at *m/e* 372, 356, and 328 are assigned as $M^+ - CH_3$, $M^+ - OCH_3$, and $M^+ - COOCH_3$, respectively, and the peak at *m/e* 271 indicates that the C-terminal amino acid is alanine. The intensive peak at *m/e* 200 corresponds to the

Table IV. Yields, Structural Data, and Tastes of Nonaromatic Acidic Oligopeptides

Fraction[a]	Yield[b]	Molar ratio(s) of AA(s)[c] *vs.* Asp or Glu	N-Terminal AA	C-Terminal AA	Mass no. (*m/e*) of main fragments	Structure	Taste and threshold,[d] mg %
A-2-2-1	12	Ser/Asp = 0.93	Ser		332 (M^+), 259, 158, 126	Ser-Asp	Flat; 200
A-3-3-1	3	Asp:Ser/Glu = 2.10:1.09		Asp		(Asp, Glu, Ser)-Asp	
B-3-1-1	4	Asp/Glu = 0.48	Glu	Glu		Glu-Asp-Glu	MSG-like; 300
B-4-1-1	47	Asp/Glu = 1.02	Glu		374 (M^+), 301, 200, 172	Glu-Asp	MSG-like; 200
B-4-2-1	2	Asp:Gly/Glu = 1.96:1.06		Asp		(Asp, Glu, Gly)-Asp	
C-2-1-1	5	Asp:Ser/Glu = 0.89:0.97	Asp	Ser		Asp-Glu-Ser	MSG-like; 300
C-3-2-1	36	Gly/Asp = 0.95	Asp		288 (M^+), 215, 186, 158	Asp-Gly	Flat; 200
D-1-1-1	28	Ala/Asp = 0.89	Asp		302 (M^+), 229, 186, 158	Asp-Ala	Flat; 200
E-4-2-1	23	Thr/Glu = 0.98	Thr		360 (M^+), 301, 172, 140	Thr-Glu	MSG-like; 300
E-5-2-2	7	Gly:Ser/Glu = 0.93:1.08	Glu	Ser	417 (M^+), 386, 271, 200	Glu-Gly-Ser	MSG-like; 200
E-6-1-1	20	Ser/Glu = 0.99	Glu		346 (M^+), 315, 200, 172	Glu-Ser	MSG-like; 200
F-1-2-1	4	Asp:Val/Glu = 1.07:1.11	Glu	Val	487 (M^+), 414, 343, 200	Glu-Asp-Val	Slightly bitter; 300
F-2-2-1	9	Val/Asp = 0.92	Val	Asp		Val-Asp	Flat; 200
F-3-1-1	78	Glu only	Glu		388 (M^+), 315, 200, 172	Glu-Glu	MSG-like; 150
F-4-1-2	6	Ser/Glu = 0.54	Ser	Glu		Ser-Glu-Glu	MSG-like; 200
F-5-2-3	2	Asp:Gly:Ser/Glu = 0.87:0.85:0.98		Glu		(Asp, Gly, Ser)-Glu	
G-4-2-1	17	Ala/Glu = 1.03	Ala		316 (M^+), 243, 128, 100	Ala-Glu	Flat; 200
G-5-1-1	21	Gly/Glu = 1.01	Glu		302 (M^+), 229, 200, 172	Glu-Gly	Flat; 200
H-1-2-1	9	Leu/Asp = 0.94	Asp		344 (M^+), 271, 186, 158	Asp-Leu	Bitter; 300
H-2-1-1	13	Ile/Asp = 0.99	Ile		344 (M^+), 271, 170, 142	Ile-Asp	Slightly bitter; 300
H-5-1-1	2	Asp:Gly:Ser/Glu = 1.72:1.08:1.05		Asp		(Asp, Glu, Gly, Ser)-Asp	
I-2-1-1	5	Asp:Ile/Glu = 1.06:1.10		Asp		(Glu, Ile)-Asp	Flat; 200
J-1-1-1	10	Val/Glu = 1.04	Val	Glu	344 (M^+), 271, 156, 128	Val-Glu	Flat; 200
J-3-2-1	6	Gln/Glu = 0.50	Glu	Glu		Glu-Gln-Glu	MSG-like; 300
K-1-1-1	17	Ile/Glu = 0.56	Ile	Glu		Ile-Glu-Glu	Slightly bitter; 300
K-3-2-2	2	Asp:Gly:Ser/Glu = 0.53:0.48:0.61		Glu		(Asp, Glu, Gly, Ser)-Glu	
L-1-2-1	14	Ile/Glu = 0.93	Ile	Glu		Ile-Glu	Slightly bitter; 300
L-2-1-1	1	Asp:Ser:Thr/Glu = 0.56:0.54:0.49		Glu		(Asp, Glu, Ser, Thr)-Glu	
M-1-1-1	1	Asp:Gly:Ser:Thr/Glu = 0.47:0.52:0.55:0.52		Thr		(Asp, Glu_2, Gly, Ser)-Thr	
N-1-2-1	4	Ile:Leu/Glu = 0.46:0.47		Glu		(Glu, Ile, Leu)-Glu	
O-2-2-1	5	Ala:Gly/Glu = 0.95:1.03			387 (M^+), 271, 200, 172[e]	Glu-Gly-Ala	Flat; 200

[a] See Table III. [b] Milligrams from 100 g of FPC. [c] Methionine and cysteine were not detected by their specific analysis (Schram *et al.*, 1954). The glutamine content (fraction J-3-2-1) was estimated from the liberated ammonia amount. [d] Each peptide was dissolved in water at pH 6.0 and the taste and threshold were evaluated by a panel of three members. [e] See Figure 3.

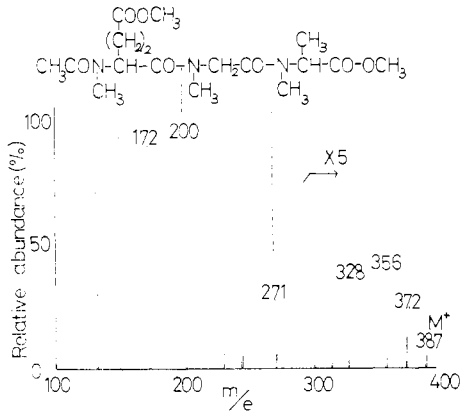

Figure 3. A representative chart of the mass spectrum for an acetyl permethyl derivative from L-glutamylglycyl-L-alanine (fraction O-2-2-1).

modified glutamyl residue and another peak at m/e 172 may show a decarbonylated fragment from this residue. Accordingly, the N terminal must be glutamic acid. The difference between 271 and 200 is in agreement with the mass number of a permethylated glycine residue. We can thus interpret the structure of this tripeptide. A similar way of interpretation applied also to other peptides listed in Table IV.

Flavor notes of these isolated peptides were evaluated by a panel of three members. It seemed to be probable that at least four dipeptides, Glu-Asp, Glu-Glu, Glu-Ser, and Thr-Glu, and five tripeptides, Asp-Glu-Ser, Glu-Asp-Glu, Glu-Gln-Glu, Glu-Gly-Ser, and Ser-Glu-Glu (threshold indicated in Table IV), had a flavor quantitatively resembling that of MSG. However, their flavor intensities were weaker than that of MSG. The threshold level of Glu-Gly-Ser, for example, was estimated to be approximately 0.2% in water at pH 5, whereas that of MSG was almost one-tenth of this level under the same conditions. Other flavor peptides were generally comparable to Glu-Gly-Ser in respect to the threshold level.

A mixture of the acidic oligopeptides, though completely free from glutamic acid, is expected to serve as a flavor potentiator which may give a more natural flavor sensation than artificial seasonings. Information obtained from the present study may give a clue to the elucidation of the flavoring activities of peptides occurring in practical fermentation foods such as soy sauces, wines, cheeses, etc.

A study is being undertaken to synthesize the isolated flavor peptides and to elucidate their individual flavor notes in more detail.

ACKNOWLEDGMENT

The authors wish to express their thanks to Tamotsu Yokotsuka, Director of Central Research Laboratories of Kikkoman Shoyu Co., Ltd., for his interest.

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Received for review February 2, 1974. Accepted September 10, 1974.

Analysis of the Lactone Fraction of Lavender Oil (*Lavandula vera* D.C.)

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The lactones extracted from lavender oil after hydrolysis were analyzed by gas chromatography and mass, infrared, and nuclear magnetic resonance spectrometry. Ten lactones were identified. Eight of these have not previously been reported as constituents of lavender oil.

In the literature concerning lavender oil about 100 components were described, among which there are only two lactones. Coumarin was already mentioned in the beginning of this century by workers of Schimmel (1900, 1903) and afterward by other investigators (Ripert, 1937; Bénezet, 1943; Seidel *et al.*, 1944). More recently Klein and Rojahn (1967) reported the presence of 4-methyl-4-vinyl-4-butanolide. This paper describes the identification of ten lactones in lavender oil.

EXPERIMENTAL SECTION

Isolation of Lactones. Lavender oil (1 kg) was added to a 5-l. flask containing a solution of 200 g of KOH (1.5 times the calculated amount based on the saponification value of the oil) in water (200 ml) and ethyl alcohol (1200 ml). The clear, homogeneous mixture was then heated for 1 hr under reflux in order to saponify the oil completely. Afterward water (1 l.) was added and the mixture was shaken vigorously. The aqueous layer, containing the potassium salts of phenols and acids and including the hydroxy acids resulting from ring opening of the corresponding lactones, was drawn off, saturated with sodium chloride, and extracted successively with ether (7 × 100 ml) and dichloromethane (3 × 100 ml) to remove the unsaponifiables. The aqueous phase was brought to pH 9.5 with 12 N H_2SO_4. The liberated phenolic compounds were removed by successive extraction with ether (1 × 100 ml,

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