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Colorimetric Detection and Determination of Carboxylic Acids with 2-Nitrophenylhydrazine Hydrochloride[1]

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Aqueous or aqueous ethanolic samples of aliphatic and aromatic acids were coupled with 2-nitrophenylhydrazine hydrochloride to give acid hydrazides, which showed intense violet colors in an alkaline medium. Dicyclohexylcarbodiimide was used as a coupling agent, and a small amount of pyridine markedly enhanced the reaction. The brown blank solution was decolorized to a faint yellow by heating the alkaline solution at 60°. The reaction conditions were investigated in detail with acetic, stearic, benzoic and citric acids, and sensitive methods for the detection and determination of carboxylic acids were established. The limits of detection and the absorption maxima of the alkaline colors obtained with many carboxylic acids were determined.

Most amino acids, together with hydroxybenzoic acids and aminobenzoic acids, were detected with reduced sensitivity by this method. Salicylic acid was shown by high-performance liquid chromatography and gas chromatography–mass fragmentography to form mainly its ethyl ester in the coupling reaction, whereas benzoic acid, which could be sensitively detected, formed only a small amount of the ester. *p*-Aminobenzoic acid also formed a large amount of its ethyl ester.

Keywords——carboxylic acids; aliphatic acids; aromatic acids; 2-nitrophenylhydrazine hydrochloride; 2-nitrophenylhydrazides; coupling reaction conditions; ethyl esters formation; colorimetry; high-performance liquid chromatography; gas chromatography-mass fragmentography

Several colorimetric methods for the detection and determination of carboxylic acids are now available which can be used directly with aqueous and/or ethanolic samples. Tanimura *et al.*[3] coupled the acids with hydroxylamine hydrochloride in the presence of dicyclohexylcarbodiimide (DCC) and detected them as the ferric hydroxamates. Kasai *et al.*[4] also developed a method for the determination of the acids.

Abbasi[5] detected the hydroxamic acids as their vanadates by colorimetry, Connors *et al.*[6] detected and determined aliphatic acids by nickel-catalyzed hydroxamic acid formation, and Johar *et al.*[7] detected carboxylic acids with acriflavine.

On the other hand, 2-nitrophenylhydrazine (2-NPH) can also be used as a coloring agent for carboxylic acids, as the acid hydrazides give intense violet colors in an alkaline medium. Legradi[8] first utilized the hydrazides for the detection of carboxylic acids by converting the acid to the acid chloride and reacting it with 2-NPH. Munson and Bilous[9] reported a method for the determination of aliphatic acids by coupling the acid with 2-NPH in the presence of DCC in a non-aqueous mixed solvent, and extracting the resulting hydrazide with aqueous

1) A part of this work was reported in a preliminary communication, *Chem. Pharm. Bull.*, **26**, 1627 (1978).
2) Location: *a*) *11, Nanakuma, Nishi-ku, Fukuoka*; *b*) *1–35, Nozawa, Setagaya-ku, Tokyo.*
3) T. Tanimura, Y. Kasai, and Z. Tamura, *Chem. Pharm. Bull.*, **20**, 1845 (1972).
4) Y. Kasai, T. Tanimura, and Z. Tamura, *Anal. Chem.*, **47**, 34 (1975).
5) S.A. Abbasi, *Mikrochim. Acta*, **1976**, 669.
6) K.A. Connors and J.W. Munson, *Anal. Chem.*, **44**, 336 (1972).
7) G.S. Johar and U. Agawara, *Talanta*, **18**, 1051 (1971).
8) L. Legradi, *Microchem. J.*, **16**, 1 (1971).
9) J.W. Munson and R. Bilous, *J. Pharm. Sci.*, **66**, 1403 (1977).

sodium hydroxide solution. The proposed method was not applied to aromatic acids, and might be tedious for aqueous samples.

We have studied the coupling reaction of carboxylic acids with 2-NPH using DCC as a coupling agent, and found that the free base reacted only with aliphatic acids, as stated by Munson *et al.* However, its hydrochloride (2-NPH·HCl) reacted sensitively with both aliphatic and aromatic acids in aqueous ethanol upon the addition of a small amount of pyridine. The blank test showed a brown color, but this decolorized to a faint yellow when the hydrazide was made alkaline and heated at 60° for 10—15 minutes. A slight turbidity which appeared in the alkaline solution was eliminated by the addition of a small amount of thiourea to the reaction mixture. Thus, a new method for the detection and determination of carboxylic acids, more sensitive than the hydroxamate methods, was established.

Experimental

Instrument——A Hitachi EPS-3T recording spectrophotometer was used for spectrophotometric measurements.

Materials——All reagents were of analytical reagent grade and were used without further purification.

Reagent Solutions——0.02 M 2-NPH·HCl Solution: Dissolve 0.380 g of the reagent in 100 ml of 99.5% ethanol by gentle warming.

Thiourea solution: Dissolve 2.0 g of the reagent in 100 ml of 99.5% ethanol.

Working 2-NPH·HCl Solution: Mix equal volumes of the above two reagent solutions before use.

Pyridine solution: Mix 7.0 ml of pyridine with sufficient 99.5% ethanol to make 100 ml.

0.25 M DCC Solution: Dissolve 0.516 g of the reagent in sufficient 99.5% ethanol to make 100 ml.

Potassium Hydroxide Solution: Dissolve 5 g of potassium hydroxide in 5 ml of water, and add sufficient methanol to make 50 ml.

Procedures a) Detection of Carboxylic Acids——Take 1 drop of an aqueous or aqueous ethanolic sample in a micro-test tube, add successively 2 drops each of 0.02 M 2-NPH·HCl solution, thiourea solution, pyridine solution and 0.25 M DCC solution. Warm at 60° for 15 min, add 1 drop of potassium hydroxide solution, and maintain at 60° for 15 min. Compare the color with the reagent blank; a violet color indicates a positive result.

b) Determination of Carboxylic Acids——Pipette 0.50 ml of an aqueous or aqueous ethanolic sample into a reaction tube of about 6 ml capacity with a screw cap,[10] and add successively 2.0 ml of working 2-NPH·HCl solution, 1.0 ml of pyridine solution and 1.0 ml of 0.25 M DCC solution. Incubate at 25° for 2 hr with the reagent blank. Add 0.50 ml of potassium hydroxide solution, cap tightly, and immerse in a water bath at 60° for 15 min. Cool in running water, and read the absorbance against the reagent blank at a suitable wavelength. Alternatively, the coupling reaction can be carried out at 37° for 1 hr, or at 60° for 20 min.

Results and Discussion

Conditions of Coupling of Carboxylic Acids with 2-NPH·HCl in the Presence of DCC

The reaction conditions were investigated with four typical carboxylic acids, namely, acetic acid (lower aliphtic), stearic acid (higher aliphatic), benzoic acid (aromatic), and citric acid (polycarboxylic). The absorption curves of alkaline solutions of the hydrazides are shown in Fig. 1; the absorption maxima are indicated in the figure legend.

The effects of reaction time on the absorbances of the developed colors are shown in Fig. 2. The reaction times used were chosen on this basis.

The concentration of 2-NPH·HCl in the reagent solution affected the absorbances of the hydrazide and of the blank solution. A more concentrated solution of the reagent gave a higher intensity, but a 0.02 M solution of the reagent was preferred for the determination because it gave an acceptable absorption in the blank (Fig. 3), and it also compensated for the reduction of absorption of amino acids by DCC. The effect of DCC concentration on the color development is shown in Fig. 4. Relatively higher absorbances were obtained in

10) The reaction tubes for a "Rapid Blood Analyser," (Chugai Seiyaku K.K., Iwamotocho, Chiyoda-ku, Tokyo) were used in this study.

the concentration range of 0.2 to 0.35 M without affecting the absorbance of the blank, and 0.25 M was selected for this study.

Pyridine might act as a catalyst in the coupling reaction with DCC. As mentioned by Munson and Bilous,[9] a small amount of pyridine significantly enhanced the absorbance of the resulting solution, but a large excess tended to reduce the intensity (Fig. 5). These experiments indicated that the optimum concentration of pyridine was 7% (v/v).

On making the hydrazide solution alkaline, a brownish insoluble substance appeared to be formed first, and the solution became slightly turbid. Addition of thiourea at a concen-

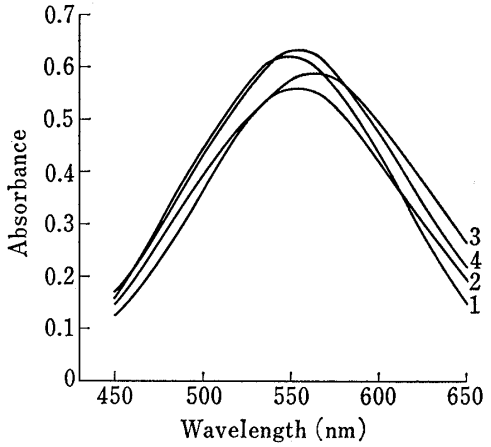

Fig. 1. Absorption Spectra of Alkaline Solutions of 2-Nitrophenylhydrazides

Each acid (0.50 μmol) was treated by the present method at 25° (see "Experimental"). Stearic acid was dissolved in 70% ethanol, and the other acids were dissolved in water.
1, acetic acid (λ_{max} 550 nm); 2, stearic acid (λ_{max} 554 nm); 3, benzoic acid (λ_{max} 565 nm); 4, citric acid (λ 554 nm).

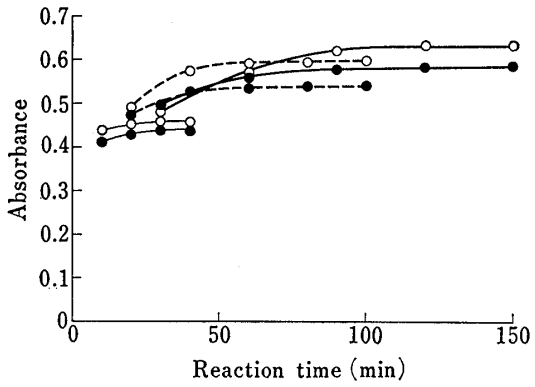

Fig. 2. Effect of the Reaction Time on the Color Development

Each acid (0.60 μmol) was treated by the standard method, changing only the reaction time.
○, acetic acid; ●, benzoic acid;
——, 25°; – – –, 37°; ——, 60°.

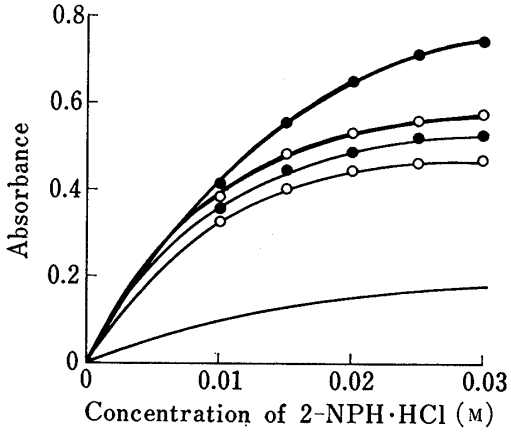

Fig. 3. Effect of the Concentration of 2-NPH·HCl on the Color Development

Each acid (0.50 μmol) was treated by the standard method at 25°, changing only the concentration of 2-NPH·HCl. The absorbance for each acid was measured at λ_{max} as indicated in Fig. 1.
—○—, acetic acid, —○—, stearic acid;
—●—, benzoic acid; —◐—, citric acid;
———, a blank test solution measured against water.

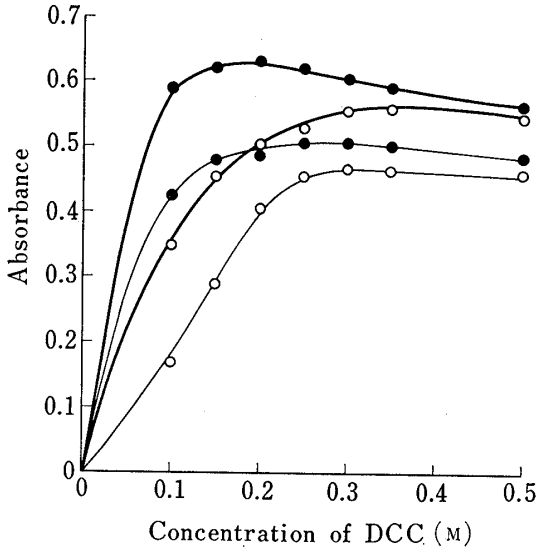

Fig. 4. Effect of the Concentration of DCC on the Color Development

Each acid (0.50 μmol) was treated by the standard method at 25°, changing only the concentration of DCC. The absorbance of each acid was measured at λ_{max} as indicated in Fig. 1.
—○—, acetic acid; —○—, stearic acid;
—●—, benzoic acid; —●—, citric acid.

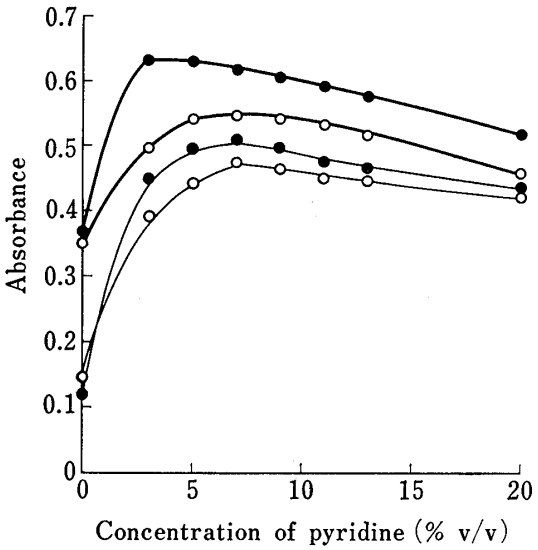

Fig. 5. Effect of the Concentration of Pyridine on the Color Development

Each acid (0.50 μmol) was treated by the standard method at 25°, changing only the concentration of pyridine. The absorbance of each acid was measured at λ_{max} as indicated in Fig. 1.
—○—, acetic acid; —○—, stearic acid;
—●—, benzoic acid; —●—, citric acid.

tration of 2% was sufficient to solubilize the unknown substance. Potassium hydroxide ionized the hydrazides, developing the violet colors. A concentration of 10% was required to decolorize the brown blank solution to a minimum and constant absorbance at 60° in 10—15 min.

The calibration plots obtained by the proposed method were linear in the range of 0.1 to 1.0 μmol of acetic, sorbic, stearic, benzoic, acetylsalicylic and phthalic acids, and in the range of 0.1 to 0.5 μmol of citric acid. When the coupling reaction was performed at 37° or 60°, the absorbances were lower than in the case of 25°. This might be due to the partial formation of unreactive ethyl esters from carboxylic acids in the coupling reaction, but the calibration plots were still linear. Three separate runs with three aliquots each at five concentrations (0.1, 0.2, 0.3, 0.4, and 0.5 μmol per 0.50 ml for citric acid, and 0.1, 0.25, 0.5, 0.75 and 1.0 μmol per 0.50 ml for the other acids) gave regression lines for which the parameters, correlation coefficients and standard errors are listed in Table I.

Table I. Parameters, Correlation Coefficients and Standard Errors of Calibration Plots[a] for Carboxylic Acids

Carboxylic acid	Temp.(°C)[b]	A	B	r[c]	S.E.[d]
Acetic	25	−0.005	1.032	0.999	0.012
	37	0.001	1.007	0.999	0.008
	60	0.002	0.780	1.000	0.008
Sorbic	25	−0.072	1.144	0.999	0.020
	37	−0.057	0.946	0.998	0.020
	60	−0.066	0.703	0.999	0.010
Stearic	25	0.002	0.906	0.997	0.015
	37	−0.033	0.908	0.999	0.014
	60	0.015	0.696	0.998	0.014
Citric	25	−0.005	1.253	0.997	0.015
	37	−0.006	1.147	0.999	0.009
	60	−0.004	0.964	0.997	0.011
Benzoic	25	−0.010	0.999	0.999	0.011
	37	−0.010	0.951	0.999	0.013
	60	−0.010	0.769	0.999	0.015
Acetylsalicylic	25	−0.019	0.800	1.000	0.008
	37	−0.003	0.742	1.000	0.007
	60	−0.004	0.564	0.999	0.009
Phthalic	25	−0.012	1.158	1.000	0.009
	37	−0.022	1.125	1.000	0.008
	60	0.002	0.992	1.000	0.011

a) Calibration plots were expressed as regression lines of the form $y=A+Bx$; y, absorbance; x, μmol of acid; A, y intercept; B, slope of the line.
b) Coupling reaction temperature.
c) Correlation coefficient of the line.
d) Standard error of the estimate (S_{xy}).

Table II. Limits of Detection and Absorption Maxima for Carboxylic Acids

Carboxylic acid	λ_{max} (nm)	Amount of acid (mol)			
		1×10^{-5}	1×10^{-6}	1×10^{-7}	1×10^{-8}
Formic	530				+[a]
Acetic	550				+
Glycolic	540				+
Trichloroacetic	364(s), 516(w)	+	±[b]		
Pyruvic	552				+
Lactic	542				+
Butyric	552				+
Sorbic	570				+
Myristic	550				+
Palmitic	550			+	±
Heptadecanoic	550				+
Stearic	554				+
Nonadecanoic	548				+
Oxalic	524	+	±		
Malonic	550				+
Succinic	553				+
Malic	548				+
Tartaric	542				+
Fumaric	373(s), 562(w)				+
Maleic	560				+
α-Ketoglutaric	442				+
Sebacic	554				+
Citric	554				+
Benzoic	565				+
o-Toluic	556				+
m-Toluic	568				+
p-Toluic	568				+
o-Chlorobenzoic	544				+
m-Chlorobenzoic	560				+
p-Chlorobenzoic	560				+
Salicylic	550	+	±		
m-Hydroxybenzoic	574				+
p-Hydroxybenzoic	598	+	±		
β-Resorcylic	356(s), 580(w)	+	±		
Acetylsalicylic	550				+
o-Aminobenzoic	362(v.s), 574(w)	+	±		
m-Aminobenzoic	570		+	±	
p-Aminobenzoic	580	+	±		
m-Nitrobenzoic	554				+
p-Nitrobenzoic	560				+
3,5-Dinitrobenzoic	358(s), 563(w)			+	±
o-Anisic	554				+
p-Anisic	572				+
Mandelic	544				+
o-Hydroxyphenylacetic	356(s), 510(w)	+	±		
p-Hydroxyphenylacetic	554				+
Cinnamic	570				+
Hippuric	540		+	±	
Nicotinic	554				+
2-Furoic	560				+
5-Nitro-2-furoic	360(s), 534(w)			+	±
Phthalic	570				+
3-Nitrophthalic	554				+

a) Limit of detection.
b) Not significant.

Table III. Limits of Detection and Absorption Maxima for Amino Acids

Amino acid	λ_{max} (nm)	Amount of acid (mol)			
		1×10^{-5}	1×10^{-6}	1×10^{-7}	1×10^{-8}
Glycine	362(s), 530(w)			+[a]	±[b]
L-α-Alanine	546		+	±	
L-Serine	360(s), 506(w)		+	±	
L-Threonine	356(s), 538(w)		+	±	
L-Valine	554		+	±	
ε-Aminocaproic acid	554		+	±	
L-Leucine	546		+	±	
L-Isoleucine	550	+	±		
L-Methionine	410				+
L-Glutamine	400(s), 534(w)		+	±	
L-Aspartic acid	386(s), 550(w)			+	±
L-Gultamic acid	538				+
L-Phenylalanine	375(v.s), 542(w)		+	±	
L-Proline	544		+	±	
L-Histidine	378(v.s), 538(w)		+	±	
L-Tryptophan	396(v.s), 470(w)		+	±	

a), *b*) are the same as in Table II.

Limits of Detection for Carboxylic Acids and Amino Acids

The detection of a variety of aliphatic and aromatic carboxylic acids could be carried out with the same reagent solutions by the procedure described in "Experimental". The limits of detection were examined by diluting stock solutions of the acids with water or aqueous ethanol. The absorption maxima were also measured by dissolving water-soluble acids in water, and water-insoluble acids in 70% ethanol. The limits of detection and the absorption maxima are given in Tables II and III.

The data in the tables show that most amino acids were less sensitively detected than other acids. This might be due to the preferential formation of unreactive acid amides relative to acid hydrazides in the coupling reaction, since DCC is widely used for peptide bond formation by the method of Sheehan and Hess.[11] Oxalic acid, which is known to decompose in the presence of DCC,[12] showed a weak response.

Esters formed by Salicylic Acid, Benzoic Acid and *p*-Aminobenzoic Acid in the Coupling Reaction

It was noteworthy that most hydroxybenzoic acids and aminobenzoic acids were also less sensitive to the color reaction, possibly because their unreactive esters might be mainly formed in the coupling procedure. To confirm this, high-performance liquid chromatography (HPLC) was carried out.

A Waters instrument equipped with a 254 nm UV detector was employed for HPLC. The column was a 30 cm×4 mm i.d. tube packed with Nucleosil 10C18. The eluents (flow rate, 1 ml/min) were methanol: water (2: 1) for ethyl salicylate and ethyl benzoate and methanol: water (1: 1) for ethyl *p*-aminobenzoate.

The coupling reaction mixtures for salicylic acid (10 μmol), benzoic acid (1.0 μmol), and *p*-aminobenzoic acid (10 μmol) were diluted with water (50 ml) and extracted with ether three times (50, 30, 30 ml). The combined ether fraction was washed successively with 10% HCl (30 ml) (except in the case of *p*-aminobenzoic acid), 5% Na_2CO_3 (20 ml) and water

11) J.C. Sheehan and G. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).
12) F. Zetzsche and A. Frendrich, *Chem. Ber.*, **72**, 363 (1939).

(20 ml), then concentrated under reduced pressure at 0°. The residues were redissolved in methanol (2—5 ml) and aliquots (10 μl) were injected into the column.

Salicylic acid gave only one peak at an elution time of 20.3 min; this was different from any other peak in the blank test. The peak was identified as ethyl salicylate by comparison with a pure standard sample. Benzoic acid and *p*-aminobenzoic acid also gave the corresponding ethyl ester peaks at elution times of 14.0 min and 12.7 min, respectively.

Ratios of Formation of Ethyl Salicylate and Ethyl Benzoate in the Reaction Mixture

It was very difficult to determine quantitatively small amounts of volatile ethyl salicylate or benzoate in the complicated coupling reaction mixture. Therefore, approximate estimations were performed to compare the ratios of formation of ethyl salicylate and benzoate by gas chromatography–mass fragmentography (GC–MF). GC–MF was performed with a JEOL JMS–D100 gas chromatograph-mass spectrometer. The column was a 100 cm×2 mm i.d. coiled glass tube packed with 15% diethyleneglycol succinate on Shimalite, 60—80 mesh. The carrier gas was helium, 2 kg/cm^2. The temperatures of the column, injection port and enricher were 200, 210 and 220°, respectively.

The residue, as a neutral fraction, was then shaken with a mixture of water (4 ml) and chloroform (10 ml), and the chloroform phase (5 ml) was separated by centrifugation. The chloroform was removed under reduced pressure at 0°, and the residues were redissolved in internal standard solutions (2—10 ml). Aliquots of the solutions (2 μl) were injected into the column. The internal standard solutions were ethyl salicylate, *m/e* 166 (retention time, 20.0 min), in chloroform (0.125 μmol/ml) for the estimation of ethyl benzoate, *m/e* 150 (retention time, 10.0 min), and ethyl benzoate in chloroform (0.3 μmol/ml) for the estimation of ethyl salicylate.

Under these conditions, the calibration curves obtained by plotting the ratio of peak areas of ethyl ester/internal standard against the concentrations of ethyl salicylate (0.2—1.0 μmol/ml) and ethyl benzoate (0.05—0.2 μmol/ml) were linear with correlation coefficients of 1.000 and 0.999, and with standard errors of 0.027 (n=5) and 0.008 (n=4), respectively.

The results showed that 10 μmol of salicylic acid in the coupling reaction gave its ethyl ester in about 96% yield at 25°, 97% yield at 37° and 98% yield at 60°. Similarly, 1.0 μmol of benzoic acid gave its ethyl ester in about 22% yield at 25°, 26% yield at 37° and 27% yield at 60°.

Thus, the insensitivity of salicylic acid in the color reaction was shown to be due to the preferential formation of the unreactive ethyl ester. The other hydroxy acids which were less sensitive to coupling might undergo similar reactions.

Aminobenzoic acids were also less sensitive to coupling, as shown in Table I. The reason for this could again be similar, since HPLC analysis of the reaction mixture of *p*-aminobenzoic acid indicated the presence of a large amount of its ethyl ester.