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STUDIES ON THE "BROWNING REACTION" OF MILK AND MILK PRODUCTS.

I. ON THE INDICATOR SPRAY FOR UREA IN FILTER PAPER PARTITION CHROMATOGRAPHY

By

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Since a few years ago it had been found that the urea-lactese system reaction is remarkable in the "browning reaction" of heated skimmilk,[1] and several studies of browning reaction in milk and milk products are being performed by the author.[2] In the investigation of the mechanisms of this system reaction by means of the filter paper partition chromatography, Ehrlich's reagent, that is to say, *p*-dimethylaminobenzaldehyde hydrochrolic acid solution has been used as the suitable indicator spray for urea-lactoside or urea-glucoside which may be produced in this system reaction.[4]

On the other hand, it has been unexpectedly known that the *p*-di methylaminobenzaldehyde reagent gives a distinctly yellow color spot of urea on the paper chromatograms,[3] and also that it can be used as an indicator spray for urea in the filter paper chromatography.

In 1948, Westall reported briefly that Ehrlich's reagent can be used in the detection of urea on the paper chromatograms. Originally, the *p*-dimethyl-aminobenzaldehyde reagent has been used in the detection of hexosamine, acethylglucosamine,[5][7] benzoic acid-glucoside, sulfamide-glucoside, sulfanilic acid-glucoside,[6] and lysine-glucoside[8] in paper chromatography. Except for Westall's report, there is no data available in detail dealing with the indicator spray for urea in filter paper partition chromatography, although the detection method of amino acid in paper chromatography has been developed.*

In this paper, the author reports on the availability of *p*-dimethylaminobenz-aldehyde reagent which is used as the indicator spray for urea in filter paper partition chromatography.

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* Recently, Williams reported that urea gives a bright green spot with sodium hypochrolide using phenol developer.[19]

Methods

Moran and Elson's method[9] used by Partridge and Westall for the detection of hexosamines was adopted for using with the filter paper techniques[5]. In this case, however, the acethylaceton reagent which is indispensable in the analysis of hexosamines is not required.

After evaporating off the elution agents, the paper strips were sprayed with the developing agents and heated in an oven for 3～5 minutes at 100°C.

Paper partition chromatography was carried out, using one-dimentional and descending boundary method. Strips (30 × 3.5 cm) were cut with a paper cutter from Toyo's No. 50 filter paper (40 × 40 cm).

p–Dimethylaminobenzaldehyde reagent : 1 g of *p*–dimethylaminobenzaldehyde which has been recrystallized twice from aqueous ethanol solution was dissolved in 30 ml of ethanol and added with 30 ml of concentrated hydrechrolic acid. Then, the solution was diluted with 200 ml of redistilled buthanol. The reagent possesses a pale yellow color and keeps for several months.

Results and Discussion

Selection of elution agents.

The urea solution is drawn into a capillary pippete whose tip is applied to the strips at the center and about 20 mm from its end. The wet area, which should not be in excess of 3 mm in diameter, is circled and allowed to dry. On every strips 20 of the urea is spotted, and the thus treated strips are inserted into the several elution agents as shown in Table 1. After the developing of the color of spot, the Rf values are measured and the color development is observed. The results obtained are shown in Table 1.

Table 1. Rf values of urea and background color with different elution agents.

Elution agents	Mixed proportion	Rf	Spet color	Background color
Methanol-water	9 : 1	0.58	Yellow	—
Ethanol-water	9 : 1	0.50	Yellow	—
Amylalcohol-water	Water saturated	0.09	Yellow	Pink violet
Buthanol-water	9 : 1	0.28	Yellow	—
Aceton		0.40	Yellow	—
Buthanol-pyridin-water	4 : 1 : 2	0.34	Yellow	Pale yellow
Lutidin-water	1 : 1	0.58	Yellow	yellow
Collidin-water	Water saturated	0.42	Yellow	yellow
Lutidin-collidin		0.06	Yellow	yellow
Buthanol-water	Water saturated	0.26	Yellow	—
Buthanol-acetic acid water	4 : 1 : 2	0.55	Yellow	—
Phenol-water	Water saturated	0.78	Yellow	—

The table shows that the buthanol containing agents is excellent in these solvents, but lutidin and collidin which show a yellow background cannot be used in respect that their colors are simillar to that of the urea. The characteristic pink violet background color appears in the use of amylalcohol and the yellow spot of urea is contrasting on this background. The solvents that are short in the elution time are as given in the following series with regard to the speed of elution :

Aceton > Lutidin > Lutidin-collidin > Methanol > Ethanol

Development of color.

To determine the specificity of this indicator spray for urea, various reagents which are usually used as developers in the paper chromatography are tested for identification of urea on the chromatograms using buthanol-acetic acid as the elution agent. The results obtained are shown in Table 2.

Table 2. Reactivity of urea on the chromoatgrams with some developing reagents.

Developing agents	Heating color condition in color development		Color development
p-Dimethylaminobenzaldehyde	100C.	30min.	+
Ninhydrin[14]	100	5	–
Ammoniak silver nitrate[17]	60	3	–
Bentidin[16]	110	5	–
Anilinhydrogenphthalate[15]	110	5	–

Table 2 shows that urea is colored only by *p*–dimethylaminobenzaldehyde.

The colored spot of urea using *p*–dimethylaminobenzaldehyde slowly becomes discolored during heating and storage of the chromatograms. The chromatograms of urea changes from yellow to dark green yellow by over heating, and from yelow to brown grey in storage for a few days. Westall reported that the color of the spot is grey, but this color must be determined with the chromatograms stored for a long time. The color of the spot is limitted to yellow as described in the above experiments.

When the papers are heated for a long time, not only the yellow spot of urea but also the paper chromatograms are changed to dark coloring, and then the papers become fragile. Therefore, the papers should be heated by the short-time low-temperature treatment.

Specificity of the method.

No color formation results when ammonium chrolide, ammonium tartarate, glycine, arginine, asparagine, aspartic acid, α–aminobuthylic acid, α–aminoisobuthylic acid, alanine, hippuric acid, guanidine carbonate, serine, threonine,

valine, methionine, uric acid, creatinine, creatine, and pyruvic acid are heated with *p*-dimethylaminobenzaldehyde reagent under prescribed conditions for urea detection, using buthanol acetic acid water (4 : 1 : 2) as the elution agent.

Lysine, cystine, histidine, thyrosine, tryptophane, and some urea derivatives, that is to say, urea-gluciside, urea-lactoside, phenylurea, sym-dimethylurea, and asym-dimethylurea give a strong color resembling the yellow of urea. Inoue, Onodera, Sisiyama, and Kitaoka reported that benzoic acid-glucoside, sulfamide-glucoside, and sulfanilic acid-glucoside give an orange color spots reacting with this reagent.[6] Glucose, galactose, fructose, alabinose, xylose, and lactose give a faint dark violet color by over heating. Perhaps many sugars will be colored by the same treatment. As these substance, however, give the characteristic Rf values differented from urea, the spot of urea can be easily determined on the chromatograms without difficult treatment. Table 3 shows the Rf values and spots of urea derivatives. These substances were synthesized by the following methods.

asym-Dimethylurea[12] : Franchimont's method
sym-Dimethylurea[14] : Fichter & Becker's method
asym-Acethylmethylurea[10] : Odenwald's method
Phenylurea[11] : Davis & Blanchard's method
sym-Diphenylurea[11] : Davis & Blanchard's method

Table 3. Rf values and spots color of the positive substance against *p*-dimethylaminobenzaldehyde reagent.

Substances	Color development	Rf	Color of spot
Urea	+	0.55	Yellow
Thiourea	+	0.85	Yellow
sym-Dimethylurea	+	0.67	Yellow
asym-Dimethylurea	+	0.65	Yellow
asym-Acethylmethylurea	−	—	—
Phenylurea	+	0.90	Yellow
asym-Diphenylurea	−	—	—

Table 3 shows that *asym*-acethylmethylurea and *asym*-diphenylurea do not give the color with this reagent, but the other substances produce the yellow spot.

Application to quantitative analysis.

10 per cent solution of urea is diluted to the solution of a certain concentration as follows in per cent :

5, 1, 0.1, 0.05, 0.01, 0.005, 0.001, 0.0005, 0.0001

Each 0.10 ml of these urea solutions is spotted on the paper strips with a capillary pippete, and the chromatography is carried out by the above mentioned method, using buthanol-acetic acid-water as the elution agent. As the colored spot of urea on the chromatograms is circled, the spot area is measured by the approximate method shown with the diameter of the spot. The results obtained are shown in Table 4. From the table it is found that the minimum detectable amount of urea by means of this method is 5γ. The relation between the amount of urea contained in a spot and the diameter of the spot on the chromatograms is also shown in Fig. 1. But the amount of urea contained in a spot is substituted the log x (x.... amount of urea) in this figure.

Table 4. Relation between the amount of urea and the spot area on the chromatograms.

Concentration of urea solution	Amount of urea contained a spot	Color develepment	diameter of a spot
0.0001 (%)	0.1 (γ)	−	— (mm)
0.0005	0.5	−	—
0.001	1	−	—
0.005	5	+	1
0.01	10	+	3
0.05	50	+	7
0.1	100	+	10
0.5	500	+	12
1	1000	+	15
5	5000	+	22

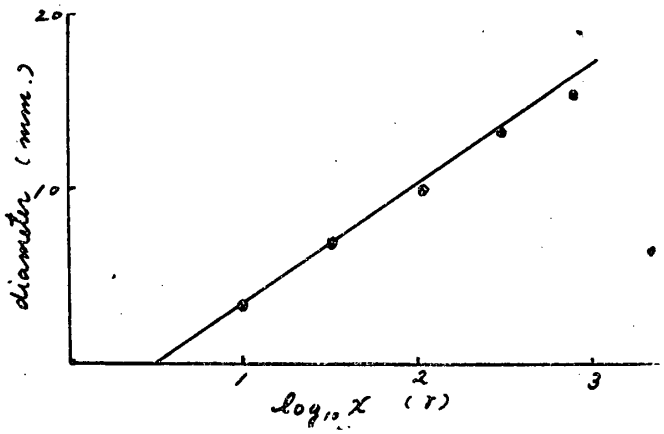

Fig. 1. Relation between the amount of urea and the diameter of the spot.

Fig. 1 shows that the relation is linear and that the approximately quantitative method of urea can be used. This method is not accurate, but is very useful in the semi-quantitative analysis of urea.

Though the method that the color is extracted from the spot urea and is compared with the color obtained from the spot of standard urea solution may give a rather more accurate value than the above method,[18] the author has not tested this colorimetric method for urea.

Summary

The filter paper partition chromatography of urea using the *p*-dimethylaminobenzaldehyde reagent as the developing agent was described, and the following results were obtained in these experiments.

1. Some solvents which are commonly used as elution agents in paper chromatography were tested for chromatography of urea and the Rf value of urea in every agent was described. As the results, elution agents in which buthanol formed the main solvent, for example, buthanol-acetic acid-water (4 : 1 : 2) was the most suitable for paper chromatography of urea.

2. The spots of urea on the chromatograms were colored by spraying *p*-dimethylaminobenzaldehyde reagent, but not by ninhydrin, ammoniak silver nitrate, benzidine, and anilinhydrogenphthalate. The color of the spot due to the *p*-dimethylaminobenzaldehyde reagent was not very stable and changed to dark yellow by over heating, and to brown grey by long time storage.

3. The urea derivatives, that is to say, thiourea, asym-dimethylurea, sym-dimethylurea, and phenylurea were colored like the urea in yellow by *p*-dimethylaminobenzaldehyde reagent, but were different in Rf values respectively. asym-Acethylmethylurea and sym-diphenylurea were not colored. Color formation resulted when tryptophane, lysine, tyrosine, histidine, and cystine were heated under the same conditions as prescribed for urea detection, but by relatively high concentration the resultant was a brown yellow spot. Many sugars were also faintly colored with this reagent by over heating.

Therefore, the spot of urea was easily detectable by the Rf value and color measurements in the mixture of these of these substance.

4. From the relation between the amount of urea and the area of the sopt which obtained the semi-quantitative analysis of urea by the determination of the diameter of the spot substituted for area measurements could be made. The minimum detectable quantities of urea in this method was 5γ.

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