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A MODIFIED SOLVENT SYSTEM AND MULTIPLE DETECTION TECHNIQUE FOR THE SEPARATION AND IDENTIFICATION OF MONO- AND OLIGOSACCHARIDES ON CELLULOSE THIN LAYERS

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SUMMARY

A method of separating oligosaccharides (DP 2–10) by means of chromatography on cellulose thin layers has been developed. The solvent system formic acid–methyl ethyl ketone–*tert.*-butanol–water (15:25:35:25) gives the best resolution for the saccharides tested, still allowing the separation of sucrose, glucose and fructose.

A multiple detection technique is also described, based upon the successive application of different spray reagents to the same plate. The utilization of the technique in the location of sugars and their identification is discussed.

INTRODUCTION

In our previous work on the soluble carbohydrates of various plants, we have achieved good separations of carbohydrate mixtures on cellulose thin layers. This technique has the advantage of both paper chromatography and of the thin-layer method, *i.e.* good resolution of biologically important carbohydrates, sufficient capacity of the layer, and ease and speed of the chromatographic procedure[1–6].

The preferred mobile phase on this support was the system formic acid–methyl ethyl ketone–*tert.*-butanol–water, first used on paper by FINK *et al.*[7] and successfully used on cellulose thin layers by VOMHOF AND TUCKER[2] for the separation of mono- and disaccharides.

With the aim of extending the use of this solvent system to the fractionation of higher DP oligosaccharides, we evaluated several systems obtained by varying the proportions of the components to give a higher polarity to the system. One of the experimental solvent systems gave excellent separations in the range of DP 2–10, while still remaining suitable for the separation of sucrose, glucose, fructose and xylose, commonly occurring sugars in higher plants.

In an attempt to improve visualization and identification of the sugar spots, we developed a technique of multiple detection, based upon the successive application of different specific sprays. The combination of two or more reagents on the same chromatogram appeared to be of value in showing up structural differences in carbohydrates. To our knowledge this technique has not been used before in the detection of sugars on thin-layer chromatograms. In the case of paper chromatograms detection

by a split reagent has been used by BAILEY AND BOURNE[8], a mixed reagent method was used by BAILEY[9], while a combination of a spray and a dip reagent has been used by DROŻDŻ AND KOWALEWSKI[10].

EXPERIMENTAL

Samples

All of the sugars used were obtained from commercial sources. The sample solutions were made by dissolving 5 mg of sugar in 2 ml of 70 % ethanol.

The oligosaccharide series were obtained by partial acid hydrolysis of starch (in 0.2 *N* H_2SO_4 at 100° for 15 min) and inulin (in 0.02 *N* H_2SO_4 at 70° for 15 min)[11], followed by neutralization with $BaCO_3$.

Preparation of the plates

20 × 20 cm plates were spread to a thickness of 0.25 mm with MN 300 cellulose (Macherey, Nagel and Co., Düren, G.F.R.) according to standard procedure with the "Chemetron" (Milan, Italy) apparatus.

The plates were allowed to dry overnight at room temperature and then stored in a desiccator cabinet.

Chromatography

Aliquots (4 μl), containing 10 μg of sugars, were spotted on the plates as superimposed spots of 0.5 μl, with a microsyringe, at 1 cm intervals and at 1.5 cm from the lower edge of the plates.

Plates were developed by the ascending technique in a 20° thermostated room.

Solvent systems

All solvents used were reagent grade obtained from E. Merck AG (Darmstadt, G.F.R.).

Various solvent systems were obtained by varying the proportions of the components of the system formic acid–methyl ethyl ketone–*tert.*-butanol–water (15:30:40:15)[2] (system A) towards a higher polarity. The preferred solvent system was found to be formic acid–methyl ethyl ketone–*tert.*-butanol–water (15:25:35:25) (system B). A threefold development with this eluent is used in order to improve separation of closely migrating saccharides[12].

The time required for a single run is approximately 3 h. No previous saturation of the chamber was found to be necessary.

Detection technique and reagents

A multiple detection technique is used which consists in the detection technique of a single chromatoplate successively with two or three reagents.

The reagents are chosen according to the following considerations:

the *initial reagent* should react specifically only with some carbohydrates;

the *intermediate reagent* (in threefold detection) should react specifically with some of the carbohydrates which have not reacted with the initial reagent;

the *final reagent* should react with all the carbohydrates or, at least, with those which have not previously reacted;

reagents which give selective colour reactions are preferred;

reagents must not give a coloured background which could interfere with the evaluation of the spots.

In the sequences of reagents tried by us the final reagent is always diphenylamine–aniline phosphate. In twofold detection this spray is preceded by a specific reagent (initial reagent). In threefold detection the best results were obtained by using the *p*-aminobenzoic acid and *p*-anisidine sprays as intermediate reagent. Colours are observed after each detection, within 5–10 min.

The following reagents gave satisfactory results:

(1) *p*-Anisidine–phthalic acid (APT)[1] (10 min at 100°).

(2) Diphenylamine–aniline–phosphoric acid (DAP) (10 min at 100°). This spray is a modified version of GALANTI's reagent[13], in which 20 ml of phosphoric acid, instead of 10 ml, are added to 100 ml of the diphenylamine–aniline solution.

(3) Dimedone–phosphoric acid (DIM)[14] (15 min at 100°).

(4) *p*-Aminobenzoic acid–phosphoric acid (PAB)[15] (15 min at 100°).

(5) Thiobarbituric acid–phosphoric acid (THB)[16] (10 min at 100°).

(6) Triphenyltetrazolium chloride–sodium hydroxide (TTC)[17] (5 min at 100°).

(7) Tetrazole blue–sodium hydroxide (TZB) (5–10 min at 40°). This spray is a modification of the tetrazole blue reagent reported by STAHL[17], in which a 1 *M* sodium hydroxide solution is used instead of the 6 *M* solution. The reduced basicity, while not significantly lowering the sensitivity of the reagent, allows the reaction of DAP with all of the sugars tested. The layer is also damaged less.

(8) Urea–hydrochloric acid (URE)[18] (40–45 min at 100°).

Other reagents tested did not give satisfactory results. Anisaldehyde reagent[17], followed by DAP, gives an intense yellow background; anthrone reagent[19] attacks the cellulose layer; α-naphthol reagent[20] also reacts with the layer, giving an intense violet colour, which interferes with the evaluation of the spots. Dimedone gives good results in the threefold detection, but it is not suitable for twofold detection with DAP.

RESULTS AND DISCUSSION

Mobile phase

The modified solvent system formic acid–methyl ethyl ketone–*tert*.-butanol–water (15:25:35:25) (system B) proved to be suitable for the fractionation of oligosaccharide mixtures, giving compact and nearly circular spots, with no tailing.

hR_G values for the sugars tested in solvent systems A and B after a threefold development are shown in Table I. The figures listed are average values of 50–60 chromatograms.

Several saccharides of the same DP have markedly different migration rates. The pairs galactose–sucrose and lactose–raffinose, which are generally difficult to separate[21], can be resolved using this system. Maltose and cellobiose also separate. Critical groups are sucrose–turanose and cellobiose–lactose–gentiobiose–melibiose.

Good separations of saccharides of different DP values are achieved. Since the migration rate decreases with increasing DP, in most cases a group separates according to DP value.

Solvent system B is most suitable for the resolution of saccharides belonging to an homologous series, *e.g.* the amylose and inulin series (Fig. 1).

TABLE I

hR_G VALUES OF MONO- AND OLIGOSACCHARIDES IN SOLVENT SYSTEMS A AND B

Sugars	*System A*		*System B*
	Threefold development	*Twofold development*[2]	*Threefold development*
2-Deoxy-D-ribose	199	—	—[a]
L-Rhamnose	180	—	122
D-Ribose	161	191	116
D-Lyxose	149	170	114
D-Xylose	143	160	113
D-Arabinose	132	145	112
D-Fructose	125	130	109
D-Mannose	120	123	104
D-Glucose	100	100	100
D-Galactose	92	91	98
Sucrose	64	65	90
Turanose	—	—	87
Maltose	—	—	76
Cellobiose	—	—	69
Melezitose	—	—	68
Lactose	—	—	68
Gentiobiose	—	—	67
Melibiose	—	—	66
Raffinose	—	—	56
Maltotriose	—	—	51
Stachyose	—	—	29

[a] Very close to the solvent front.

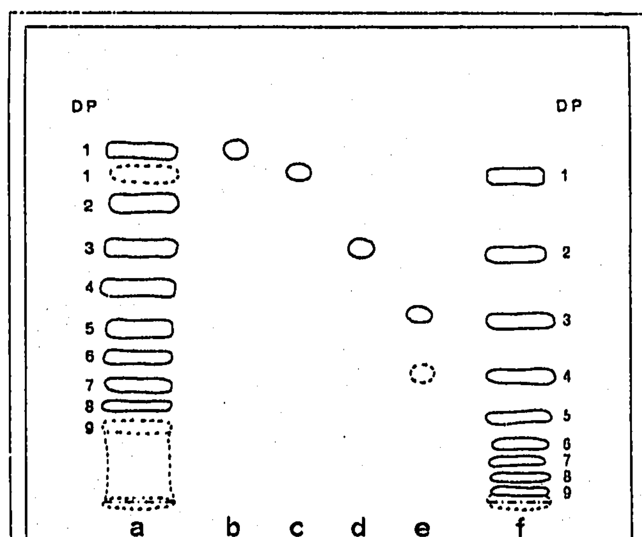

Fig. 1. Separation on a cellulose layer with solvent system B, three elutions of 15–16 cm; the spots were detected with APT followed by DAP. (a) Inulin hydrolysate; (b) fructose; (c) glucose; (d) maltose; (e) maltotriose with traces of maltotetraose found in one of the samples used; (f) starch hydrolysate.

Multiple detection

The results obtained with the various sequences of reagents chosen for application to the multiple detection technique are listed in Tables II and III. The results reported were obtained with a standard amount of 10 μg of each sugar.

The visualization limit in the final detection is generally 0.5–1 μg for monosaccharides and sucrose, and 1–2 μg for other oligosaccharides. Maximum sensitivity is observed for pentoses (0.1–0.2 μg), when the initial reagent is APT or PAB, and for fructose and sucrose (0.1–0.2 μg), when the initial reagent is THB. A higher detection limit is exhibited by cellobiose (2–4 μg).

With all the sugars examined, the nature of the colours obtained is independent of sugar concentration over the range 0.1–10 μg.

Twofold detection. In twofold detection the pairs of reagents which were found to be most suitable are listed in Table II.

In the initial step, 2-deoxyribose is visualized only by APT and TTC; rhamnose by APT, PAB and TTC. Aldopentoses (ribose, lyxose, xylose, arabinose) react with all the reagents tested, with the exception of THB and URE, which are specific for free and linked ketose units. Generally the colours obtained are not distinctive for the single pentoses, but they clearly differentiate the pentoses from other classes of sugars. Similar behaviour is found for the aldohexoses (mannose, glucose, galactose), which do not react with THB, URE and TZB. Fructose reacts with all the reagents tested, the one exception being APT.

The speed of colour development of fructose is generally higher than that of the aldoses.

Sucrose and turanose, which in solvent B migrate at approximately the same rate, give the typical colour reaction of ketose containing saccharides with URE and THB. While no differentiation between the two sugars is afforded with URE, a slight difference is observed with THB, with which sucrose is yellow-orange and turanose is yellow. Under the conditions used, the two disaccharides do not react with APT, whilst only sucrose is visualized by PAB.

Maltose and cellobiose, which differ only in the configuration of glycosidic bonds, show a different behaviour with APT and PAB: under the conditions used, only cellobiose is detected. Neither saccharide reacts with TZB. Maltotriose behaves like maltose.

Lactose, gentiobiose and melibiose may be differentiated through their reaction with APT (no reaction with melibiose) and PAB (lactose, yellow; gentiobiose and melibiose, yellow-brown).

Melezitose, which migrates together with cellobiose, lactose, gentiobiose and melibiose, is easily distinguished by being non-reducing (TTC negative) and by containing a fructose unit (THB and URE positive).

Raffinose and stachyose, both sucrose galactosides, behave as melezitose; their terminal fructose unit reacts more rapidly with URE than the internal fructose unit of melezitose.

In the final detection all the sugars react, while the initial colours are modified allowing further differentiation. Epimeric pentose pairs (ribose and arabinose, lyxose and xylose) are differentiated with APT + DAP and THB + DAP. With TZB + DAP and URE + DAP a further differentiation of ribose from the other aldopentose is obtained. Among the aldohexoses, galactose gives a different colour than the epimer

TABLE II

COLOUR REACTIONS OF MONO- AND OLIGOSACCHARIDES ON CELLULOSE THIN LAYERS IN TWOFOLD SPRAY

A = aldose; K = ketose; R = reducing; NR = non-reducing; Bk = brick; Bl = blue; Br = brown; V = violet; W = white; Y = yellow; F = faint; P = pale; Lg = light; In = intense.

Sugars	*R/NR*	*A/K*	*DAP*	*APT*	*APT + DAP*	*PAB*	*PAB + DAP*
2-Deoxyribose	R	A	Pk	Y	Pu	—	Pk Br
Rhamnose	R	A	Y Gr	Y	Y Gr	Y	Y Gr
Ribose	R	A	Ol	Pk to Bk R	Br Y	Bk R	R Br
Lyxose	R	A	Ol	Pk to Bk R	Br Gy	Bk R	R Br
Xylose	R	A	Ol	Pk to Bk R	Br Gy	Bk R	R Br
Arabinose	R	A	Ol	Pk to Bk R	Br	Bk R	R Br
Fructose	R	K	Y Or	—	Y	Y	Y Br
Mannose	R	A	Bl Gy	Y	Gy	Y Br	Br Gy
Glucose	R	A	Gy Bl	Y	Gy	Br Y	Br
Galactose	R	A	Gy	Y	Ol	Br Y	Br
Sucrose	NR	A-K	Br Y	—	Y Gy	P Y	Br Gy
Turanose	R	A-K	Y Br	—	Br Gy	—	Gy V
Maltose	R	A-A	Bl	—	Bl	—	Bl Gy
Cellobiose	R	A-A	Bl	Y Br	Gy Bl	Y	Gy Gr
Melezitose	NR	A-K-A	Y Gy	—	Gy Y	—	Gy Y
Lactose	R	A-A	Gy Bl	F Y	Gy Bl	Y	Gy Gr
Gentiobiose	R	A-A	Gy Bl	Y	Gy	Y Br	Br Gy
Melibiose	R	A-A	Gy Bl	Y	Gy	Y Br	Br
Raffinose	NR	A-A-K	Y Gy	—	Y Gy	—	Gy Y
Maltotriose	R	A-A-A	Gy Bl	—	Bl	—	Bl Gy
Stachyose	NR	A-A-A-K	Y Gy	—	Y Gy	—	Gy Y
Background	—	—	W	W	F Gy	W	F Gy

mannose and glucose with APT + DAP (galactose grey, mannose and glucose olive) and with THB + DAP (galactose grey-yellow, mannose and glucose grey-brown).

Sucrose and turanose give markedly different colours with APT + DAP, PAB + DAP, TTC + DAP and TZB + DAP. PAB + DAP and TTC + DAP also differentiate maltose from cellobiose. Differences are observed between lactose, gentiobiose and melibiose upon treatment with PAB + DAP. As could be expected, all the pairs of reagents tested give similar colours with melezitose, raffinose and stachyose.

Threefold detection. In threefold detection the best results were obtained with the sequences DIM + PAB + DAP, THB + PAB + DAP and URE + PAB + DAP. In these sequences, PAB can be replaced by APT. The colours are listed in Table III.

The successive detection with DIM, PAB, DAP provides a selective visualization of the sugars tested. This sequence allows discrimination between cellobiose and maltose and between lactose, gentiobiose and melibiose. It is noteworthy that maltose does not react with the intermediate reagent, whereas cellobiose does, thus providing further differentiation between the α and the β configuration of the $1 \rightarrow 4$ linkage.

If APT is used as intermediate reagent instead of PAB, the final detection with DAP allows glucose and galactose to be distinguished.

In the THB + PAB + DAP sequence, the first spray gives a positive reaction

DETECTION
Gr = green; Gy = grey; Lc = lilac; Ol = olive; Or = orange; Pk = pink; Pu = purple; R = red;

THB	*THB + DAP*	*TTC*	*TTC + DAP*	*TZB*	*TZB + DAP*	*URE*	*URE + DAP*
—	Lc	Pk	Pu	—	Pk	—	Br
—	Y Gr	Pk	Y Br	—	Y	—	Gy Br
—	Gy Bl	In Pk	In Pk	Lc	Gy	—	Pk
—	Gr	In Pk	In Pk	F Lc	Gy Ol	—	Or Pk
—	Gr	In Pk	In Pk	F Lc	Ol	—	Or Pk
—	Gy Gr	In Pk	In Pk	F Lc	Gy Ol	—	Or Pk
Y Or	In Y Br	In Pk	Bk R	In Lc	Gy Pu	Gy Bl	Gy Bl
—	Gy Br	Pk	Gy Pk	—	Gy	—	Lc
—	Gy Br	Pk	Gy Pk	—	Gy	—	Lc
—	Gy Y	Pk	Pk Gy	—	Gy	—	Lc
Y Or	In Y Br	—	Y Br	—	Y	Gy Bl	Gy Bl
Y	In Y Br	In Pk	Pk Gy	In Lc	Gy V	Gy Bl	Gy Bl
—	Y Br	Pk	Lc Gy	—	Bl	—	F Gy Bl
—	Y	Pk	Gy	—	Bl	—	V
Y	In Y Br	—	Y Gy	—	Gy Y	F Gy Bl	Gy
—	Y	Pk	Pk Gy	—	Bl	—	V
—	Y Gr	Pk	Pk Gy	—	Gy Bl	—	Lc
—	Y Gr	Pk	Pk Gy	—	Gy Bl	—	Lc
Y	In Y Br	—	Y	—	Y	Gy Bl	Gy
—	Y Br	Pk	Lc Gy	—	Bl	—	F Gy Bl
Y	In Y Br	—	Y	—	Y	Gy Bl	Gy
W	F Y Gr	F Pk	F Pk Gr	F Lc	F Gy	W	W

with free and linked ketose units. The second spray results in the appearance of spots for aldopentoses, aldohexoses and a few oligosaccharides, while maltose, higher homologues of the maltose series and lactose do not react. All the sugars are clearly visible after the last spray, with specific colours. Differentiation between sucrose and turanose and between $1 \rightarrow 4$ and $1 \rightarrow 6$ linked aldohexose disaccharides is possible.

When using APT instead of PAB, ribose and arabinose give a green-yellow colour, while lyxose and xylose are green. Cellobiose and melezitose are also differentiated.

In the URE + PAB + DAP sequence, URE reacts specifically with ketose-containing sugars. When the ketose unit is non-terminal, as in melezitose, only a faint colour is obtained. After spraying with PAB, all the sugars, except deoxyribose and maltose, react. Sucrose and turanose are differentiated at this step. After spraying with DAP, deoxyribose and maltose can also be detected. With this sequence of reagents ribose shows a reddish brown colour, different from the brown-orange colour of the other aldopentoses. If PAB is replaced by APT, arabinose, after the third spray, is slightly different in colour from the other aldopentoses. In addition, lactose is grey-brown, while gentiobiose and melibiose are lilac-grey.

As a rule, APT gives more greyish colours than PAB when used as intermediate reagent.

Slight variations in colours may be occasionally noted, connected with the

TABLE III

COLOUR REACTIONS OF MONO- AND OLIGOSACCHARIDES ON CELLULOSE THIN LAYERS IN THREEFOLD SPRAY

A = aldose; K = ketose; R = reducing; NR = non-reducing; Bk = brick; Bl = blue; Br = brown;
V = Violet; W = white; Y = yellow; F = faint; P = pale; Lg = light; In = intense.

Sugars	*R/NR*	*A/K*	*DIM*	*DIM + APT*	*DIM + APT + DAP*	*DIM + PAB*	*DIM + PAB + DAP*	*THB*
2-Deoxyribose	R	A	Lc	Gy V	V	Gy V	Gy V	—
Rhamnose	R	A	—	Y	Y	Y	Y Gy	—
Ribose	R	A	—	Or Br	Y Br	Or Br	Br Gy	—
Lyxose	R	A	—	Or Br	Y Br	Or Br	Br Gy	—
Xylose	R	A	—	Or Br	Y Br	Or Br	Br Gy	—
Arabinose	R	A	—	Or Br	Y Br	Or Br	Br Gy	—
Fructose	R	K	Gr Y	Y Gr	Y Gr	Y	Y Gy	Y Or
Mannose	R	A	—	Y Br	Gy Y	Pu	Br	—
Glucose	R	A	—	Y Br	Gy Y	Pu	Br	—
Galactose	R	A	—	Y Br	Y Br	Pu	Br	—
Sucrose	NR	A-K	Gr Y	Y Gr	Y Gr	Br	Br Y	Y Or
Turanose	R	A-K	Y Gr	Y Gr	Y Gr	Br Y	Br Y	Y
Maltose	R	A-A	—	F Y	Bl	—	Bl	—
Cellobiose	R	A-A	—	F Y	Gy Bl	Pu	Gy	—
Melezitose	NR	A-K-A	Y	Y	Y Gr	Br Y	Br	Y
Lactose	R	A-A	—	F Y	Gy Bl	Pu	Gy	—
Gentiobiose	R	A-A	—	Y Br	Gy Y	Pu Y	Gy Br	—
Melibiose	R	A-A	—	Y Br	Gy Y	Or Br	Gy Br	—
Raffinose	NR	A-A-K	Y	Y	Y Gr	Y Br	Y Br	Y
Maltotriose	R	A-A-A	—	F Y	Bl	—	Bl	—
Stachyose	NR	A-A-A-K	Y	Y	Y Gr	Y Br	Y Br	Y
Background	—	—	W	Lg Y	Lg Y	Pk Y	Lg Gy	W

inevitable variations in spraying intensity, heating time and temperature. Accurate standardization of detection procedure is therefore very important for reproducibility of colours.

Other spray reagents for the detection of sugars should also prove applicable to the technique described.

The methods reported are already in use in our laboratory and have proved to be of value in studies on oligosaccharides. The same sequences of reagents have also been applied by us on silica gel layers with satisfactory results.

REFERENCES

1 A. Schweiger, *J. Chromatogr.*, 9 (1962) 374.
2 D. W. Vomhof and T. C. Tucker, *J. Chromatogr.*, 17 (1965) 300.
3 M. L. Wolfrom, D. L. Patin and R. M. de Lederkremer, *J. Chromatogr.*, 17 (1965) 488.
4 M. L. Wolfrom, R. M. de Lederkremer and G. Schwab, *J. Chromatogr.*, 22 (1966) 474.
5 D. W. Vomhof, J. Truitt and T. C. Tucker, *J. Chromatogr.*, 21 (1966) 335.
6 M. Duckworth and W. Yaphe, *J. Chromatogr.*, 49 (1970) 482.
7 K. Fink, R. E. Cline and R. M. Fink, *Anal. Chem.*, 35 (1963) 389.
8 R. W. Bailey and B. J. Bourne, *J. Chromatogr.*, 4 (1960) 206.
9 R. W. Bailey, *J. Chromatogr.*, 8 (1962) 57.

DETECTION

Gr = green; Gy = grey; Lc = lilac; Ol = olive; Or = orange; Pk = pink; Pu = purple; R = red;

THB + APT	*THB + APT + DAP*	*THB + PAB*	*THB + PAB + DAP*	*URE*	*URE + APT*	*URE + APT + DAP*	*URE + PAB*	*URE + PAB + DAP*
—	Lc	—	Pk	—	—	V Gy	—	Br Gy
Y	Y	In Y	Y Br	—	Y	Y Gy	Y	Br Y
Gr Y	Gy Bl	R Br	R Br	—	Pk to Bk R	Pk Br	Pk Or	R Br
Gr	Gy Gr	R Br	R Br	—	Pk to Bk R	Pk Br	Pk Or	Br Or
Gr	Gy Gr	R Br	R Br	—	Pk to Bk R	Pk Br	Pk Or	Br Or
Gr Y	Gy Bl	R Br	R Br	—	Pk to Bk R	Pk Or	Pk Or	Br Or
Or	Or	In Y	Y Br	Gy Bl	Ol	Ol	Bl Gr	Bl Gy
Y Or	Y Gr	Or	Br Gy	—	F Y	Gy Br	Y Gy	Gy V
Y Or	Ol	In Or	Br	—	Y	Gy Br	Y Gy	Gy V
Y Or	Y Gr	Or	Y Br	—	Y	Gy Br	Y Gy	Gy
Or	Y Br	Y Br	Br	Gy Bl	Gy Y	Ol	Ol	Gy Bl
Y Or	Y Br	F Y	Pk Gy	Gy Bl	Y Gy	Gy	Y Gy	Gy
—	Gy Y	—	Gy Bl	—	—	F Bl Gy	—	F Gy
F Y	Y Gy	F Y	Gy	—	F Y	Y Gy	F Y Gy	Gy
Y Or	Y Br	Y	Y Pk	F Gy Bl	Y Gy	Gy	Y Gy	Gy
F Y	Y Gy	—	Gy	—	F Y	Gy Br	F Y Gy	F Gy
F Y	Ol	F Or	Br Gy	—	F Y	Lc Gy	F Y	Gy V
F Y	Gr Y	F Or	Gy Br	—	F Y	Lc Gy	F Y	Gy V
Or	Y Br	Y	Br	Gy Bl	Ol	Ol	Ol	Gy Bl
—	Gy Y	—	Gy Bl	—	—	F Bl Gy	—	F Gy
Or	Y Br	Y	Br	Gy Bl	Ol	Ol	Gy Y	Gy Bl
Lg Y	Y Gr	Lg Y	Lg Gr	W	W	P Gy	W	W

10 B. Drożdż and Z. Kowalewski, *J. Chromatogr.*, 20 (1965) 188.
11 D. French and G. M. Wild, *J. Amer. Chem. Soc.*, 75 (1953) 2612.
12 J. A. Thoma, *Anal. Chem.*, 35 (1963) 214.
13 M. Galanti, *Bull. Soc. Chim. Biol.*, 40 (1958) 265.
14 S. Adachi, *Anal. Biochem.*, 9 (1964) 224.
15 A. S. Saini, *J. Chromatogr.*, 24 (1966) 484.
16 F. Percheron, *Bull. Soc. Chim. Biol.*, 44 (1962) 1161.
17 E. Stahl, *Dünnschicht-Chromatographie*, 2. Auflage, Springer Verlag, Berlin, 1967, pp. 815–856.
18 R. Dedonder, *Bull. Soc. Chim. Biol.*, 34 (1952) 144.
19 R. Johanson, *Nature*, 172 (1953) 956.
20 H. Jacin and A. R. Mishkin, *J. Chromatogr.*, 18 (1965) 170.
21 S. Clermont-Beaugiraud, *Qual. Plant. Mater. Veg.*, 16 (1968) 92.