ELSEVIER

Carbohydrate Polymers 37 (1998) 263–272

Carbohydrate Polymers

Application of planar chromatography to sample analysis in the sugar industry

G. Mantovani*, G. Vaccari, E. Dosi, G. Lodi

Chemistry Department, University, Via L. Borsari 46, I-44100 Ferrara, Italy

Received 8 December 1997

Abstract

Analysis of sugar mixtures by modern liquid chromatographic techniques, both column and planar, has gained in prominence. As far as planar chromatography is concerned, the introduction of high performance bonded-phase layers and instrumental development techniques has opened up new avenues in the TLC analysis of sugars, especially for samples that normally require cumbersome clean-up processes. In chromatography, gradient elution is the technique of choice when the mixture components span a wide range of polarities (or molecular structure). Planar chromatography gradient development is also possible using an automated technique (AMD), which has been recently introduced. This technique makes large spot capacities possible because of the reconcentration effect caused by the multiple development as well as the accommodation of many spots on the same chromatographic plate, because of the gradient development. Thus, complex samples, such as beet or cane molasses, can be analyzed (or at least screened) on high-performance thin layers using modern scanners which allow a great deal of information to be obtained. In the analysis of molasses, over pressure liquid chromatography (OPLC) is also interesting. This is a forced flow technique which makes both large spot capacities and short development times possible, and can be utilized for gradient elutions as well. Details of the analysis of both beet and cane molasses are reported, and a comparison between the two techniques mentioned previously is discussed.

Keywords: Planar chromatography; Oligosaccharides; Sugar industry

1. Preamble

The acquisition and utilization of an analytical technique in quality control laboratories, factory processing control laboratories, and in general in the applied research field, depend upon some requirements which must be fulfilled by the technique itself. Such requirements may be summarized as follows:

- rapidity of analysis;
- simplest pretreatment;
- automation of the various steps;
- reproducibility of results;
- possibility of simultaneously analyzing a number of samples;
- possibility of simultaneously evaluating a number of analytical parameters;
- limited cost of instruments and their maintenance;
- limited cost of each single analysis;
- possibility of utilizing operators who are not highly skilled;
- very large range of products which can be analyzed;
- utilization of non-toxic or non-polluting reactants;
- possibility that via such a technique official methods of analysis can be established.

Obviously, it is difficult for an analytical technique to conform to all the aforementioned requirements. Therefore, we will try to evaluate what modern planar chromatography can offer with respect to the determination of carbohydrates which are present in real matrixes and, in particular, those of the cane or beet sugar industries.

2. Introduction

The first separation of sugars present in a mixture using planar chromatography was described by Partridge in 1946 (Partridge, 1965) utilizing paper chromatography. In 1961 Stahl and Kaltenbach (1961) published the first results of separation of monosaccharides by thin layer chromatography (TLC). From then on, many papers have been published utilizing TLC for the determination of mono-, di-, oligo- and poly-saccharides present in juices, molasses and raw sugars

* Corresponding author

0144–8617/98/$ - see front matter
PII: S0144-8617(98)00069-1

from both cane and beet, with the result that some methods have even been considered by the I.C.U.M.S.A. (International Commission for Uniform Methods of Sugar Analysis) (Schneider, 1979). In 1975, the first application of high performance liquid chromatography (HPLC) to the separation of sugars utilizing an aqueous mobile phase was reported (Linden and Lawhead, 1975; Goulding, 1975). Then use of the HPLC technique expanded rapidly, and in so doing, developed various branches according to the type of solid phase or the detector adopted. Such a development was possible thanks to continuously evolving instrumental techniques which, until few years ago, did not include planar chromatography and which, consequently, seemed to have lost its appeal. Even the I.C.U.M.S.A., in l990 (ICUMSA, 1990), pointed out that TLC had been superseded by more modern methods such as HPLC.

For a number of years now, interest in planar chromatography seems to have been revived, either by the introduction of high performance layers (HPTLC) or by the introduction of instrumental techniques that have been perfected and are completely automated. Such techniques cover all the various analytical phases starting from the positioning of the samples on the plate, to their elution, development and their qualitative or quantitative evaluation.

3. The new HPTLC layers

In Table 1 the characteristics of the new HPTLC layers are compared with those given by the conventional layers. A narrow distribution of the sizes of the particles and a remarkable reduction in their sizes, as clearly shown by the micrographs in Fig. 1, make it possible to obtain layers having high performances. Such new layers require smaller amounts of sample and shorter migration distances thus making high separation potentials achievable, saving analysis time with better resolutions and lower detection limits.

Table 1
Comparison of conventional TLC plates with HPTLC plates

Parameter	Conventional TLC	HPTLC
Plate size	20 × 20 cm	10 × 10 cm 10 × 20 cm
Layer thickness	100–200 μm	200 μm
Particle size: average	20 μm	5–15 μm
distribution	10–60 μm	Narrow
Sample volume	1–5 μl	0.1–0.2 μl
Starting point diameter	3–6 mm	1.0–1.5 mm
Separate point diameter	6–15 mm	2–6 mm
Solvent migration distance	10–15 cm	3–6 cm
Time for development	30–200 min	3–20 min
Detection limits: absorbance	1–5 ng	0.1–0.5 ng
Fluorescence	0.05–0.1 ng	0.005–0.01 ng
Tracks per plates	10	18 or 36

Fig. 1. Micrographic comparison of traditional TLC plates (a), with HPTLC plates (b).

4. Modern planar chromatography

As well utilizing high performance layers, planar chromatography now makes use of efficient and automated chromatographic elution systems. In Fig. 2 the more common elution methods are reported.

In the first part of the present paper, dealing with the separation and determination of carbohydrates in complex matrixes, we consider HPTLC and development of the automated method AMD (see later), whilst in the second part of the paper some results relating to utilization of the OPLC technique are reported. The completely automated densitometric technique was utilized for qualitative evaluation. Before describing in detail the techniques of elution adopted, we will describe the technique for positioning the samples.

5. The technique for positioning the samples

The samples to be analyzed, which generally have to be simply diluted and then filtered through disposable filters if they are highly turbid, must be applied to the layer as spots of small diameters or sufficiently thin bands, with the aim of

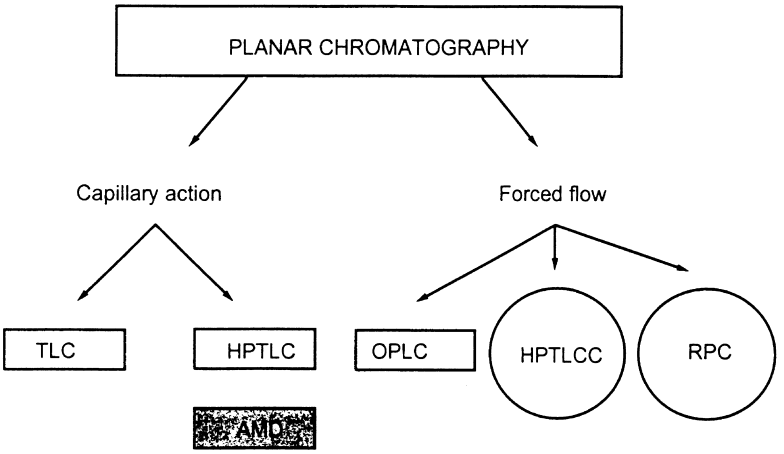

Fig. 2. Different elution methods in planar chromatography. TLC, thin layer chromatography; HPTLC, high performance thin layer chromatography; OPLC, over pressure layer chromatography; HPTLCC, high performance thin layer circular chromatography; RPC, rotational planar chromatography; and AMD, automated multiple development.

not compromising the layer efficiency. The amount of sample must be limited to some microlitres to avoid an overloading of volume or mass. Moreover, a known amount of sample must be applied at a precise point. There are various automatic devices which make it possible to place spots or bands having such characteristics. A type used at present mechanically moves the plate below a fixed syringe while following a fixed programme. An atomizer sprays the sample via a nitrogen flow from the syringe thus forming a very thin band on the plate surface. The thin bands can be easily located on the plate for the densitometric determination. The reproducibility of the sample volume placed on the plate is very high and the whole operation requires only a few minutes.

6. The technique of automated multiple development (AMD)

The most efficient method for increasing the resolution under development conditions using a capillary flow is based on the utilization of the multiple development. Planar chromatography provides mono- and bi-dimensional separations. In the case of mono-dimensional multiple

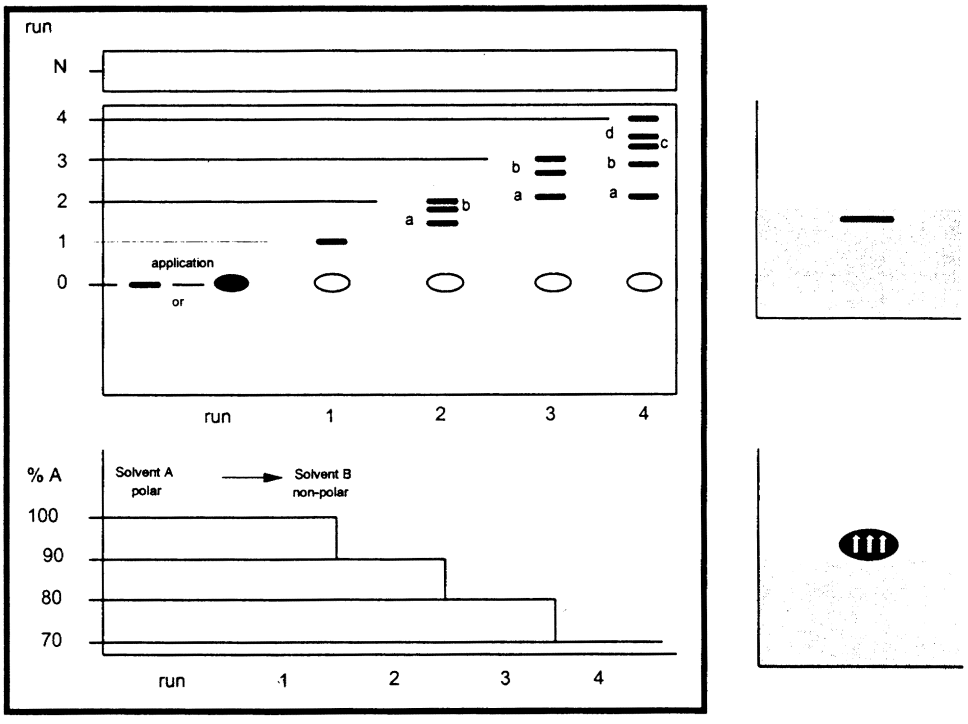

Fig. 3. Mechanism of reconcentration of the spot in order to limit the diffusion of the zone itself as a function of the solvent polarity.

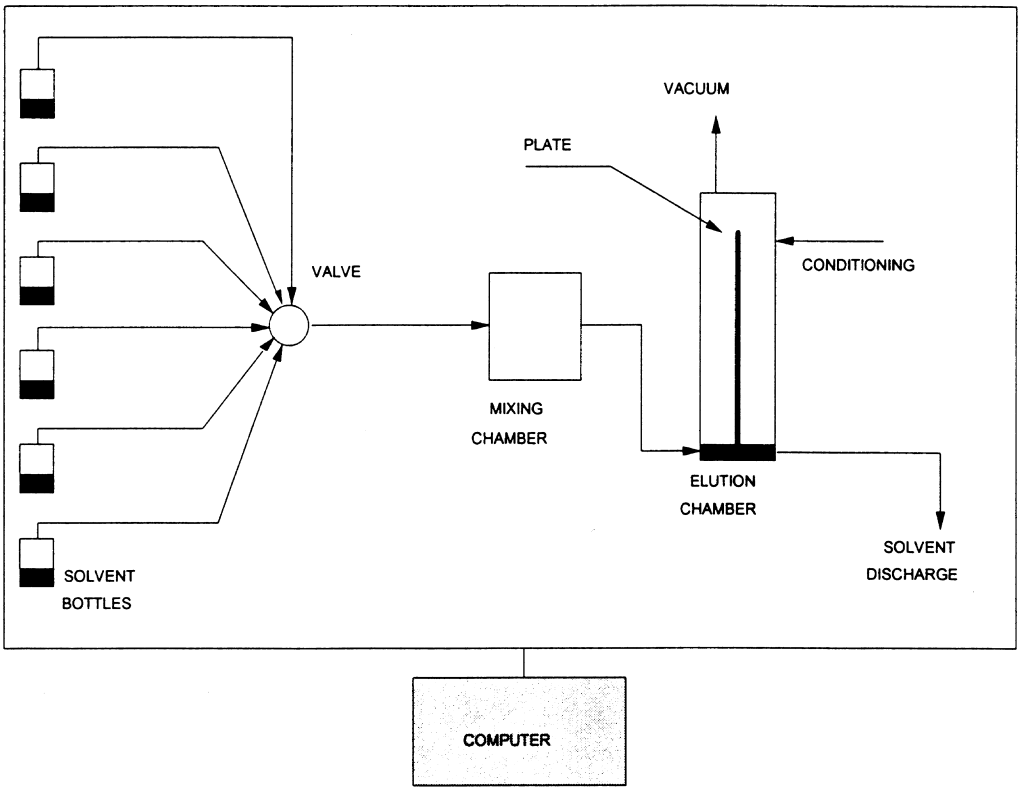

Fig. 4. Basic scheme of the AMD technique.

development, the TLC plate is developed along a selected certain distance or time. Then the development is stopped and the solvent removed by evaporation. The development step is automatically repeated a number of times. Such a strategy for the separation of complex mixtures allows for variations in the primary experimental parameters: the length of the chromatographic plate, the composition of the mobile phase in each development step and the number of developments. Another particular characteristic of the multiple development process is the mechanism of reconcentration of the zone (Fig. 3) which increases the efficiency of the layer by hindering the diffusion process of the zone itself.

Each time the solvent crosses the solid phase, it compresses the spots in the direction of the development. Thus the spot, if initially round, firstly becomes oval and finally becomes a thin band if a sufficient number of development steps have been utilized. The compression occurs because the mobile phase firstly meets the inferior portion of the spots, were the molecules of the samples begin to move, before the molecules ahead of the solvent front. When the front of the solvent passes through the reconcentrated band, the latter migrates and spreads by diffusion, following the usual mechanism. Therefore, it is possible to promote the migration of a spot for a considerable distance without greatly changing its dimensions in the direction of migration. In order that the effect of the band reconcentration is appreciable, it is necessary that the number of the steps is adequate, so that automation of the whole process becomes indispensable.

The process of automated multiple development (AMD) is now possible by utilizing the apparatus with the operational scheme as depicted in Fig. 4.

For the separation of sugars we have used the CAMAG–AMD instrument (Muttenz, Switzerland). The AMD operating sequence begins with nitrogen drying which involves the evacuation of the sealed developing chamber. This is followed by a conditioning step to control the layer activity. To commence development, a mixing valve selects the initial solvent composition from the solvents available in the mixing chamber. At the appropriate time a fixed mobile phase volume is forced into the developing chamber and a chromatographic separation developed for a pre-selected distance. The mobile phase is then sucked from the developing chamber into a waste solvent bottle and the solvent vapours evacuated from the chamber by means of a vacuum pump. After the drying step, the next cycle commences with the conditioning of the layer, introduction of fresh mobile phase, removal of the mobile phase at the end of the selected development time, again followed by vacuum drying of the layer. All the processes are automated and time-sequenced so that when a program has been entered into the control unit it can be executed without operator intervention. Depending upon the number of developments scheduled, the cycle can proceed for several hours and can even be carried out overnight, bearing in mind the complete automatation of the system.

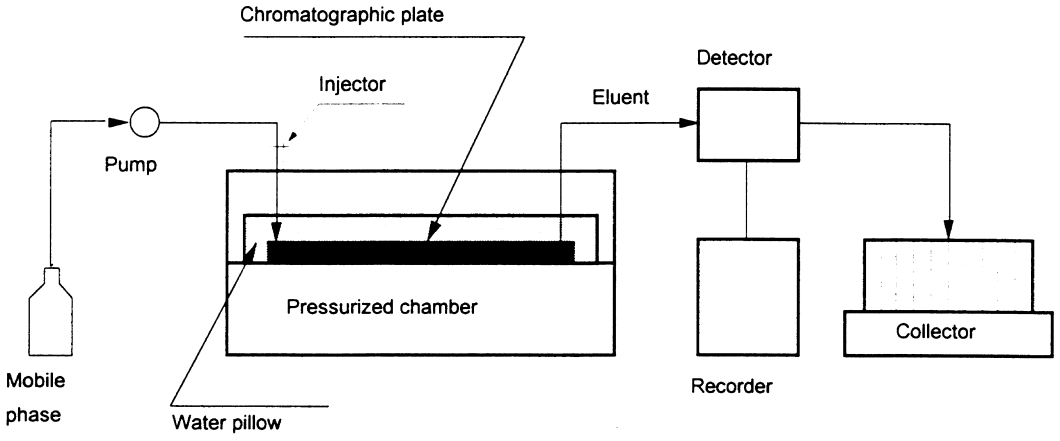

Fig. 5. Basic scheme of the OPLC technique.

7. The OPLC technique

The OPLC technique, developed by Tyihak et al. (Tyihak and Minczovics, 1979) is a planar technique using elution under pressure which includes the advantages of TLC and HPTLC classical chromatography as well as those given by column chromatography (HPLC). As in column chromatography, in OPLC the vapour phase has been eliminated because the layer is completely covered by an elastic membrane under an external pressure. Using a pump (Fig. 5) the mobile phase is eluted through the porous layer. The thin layer then behaves as a planar column which can work off-line or on-line. The possibility of using high pressure in the pillow makes it possible to use mobile phases having relatively high viscosities. We point out that the plates used must have a particular 'rim', which can be easily set up even in the laboratory on normal plates.

8. Derivatization

The plates, after AMD or OPLC elution, depending upon the characteristics of the layer, can be derivatized via direct heating in an oven for a few minutes or treated in advance with a suitable reagent. Even this operation can be carried out very rapidly using an automated device which dips the plate into the derivatization solution.

9. Quantitative determination via densitometry

Thin layer chromatography could not be compared with other chromatographic techniques in the absence of the availability of instruments capable of quantifying the planar separations in situ. Nowadays, we can find on the market suitable scanners which, within a few seconds, make it possible to transfer the results of separations on a plate to absorption spectra, thus giving quantitative data for the separated components. We have utilized a SCANNER III-CAMAG interfaced with a computer which is managed by CATS 4.03 software. As far as determination of sugars is concerned, the plates were scanned for fluorescence (excitation at 365 nm (Hg), filter cut-off 400 nm) or for absorbance ($\lambda_{max} = 515$ nm).

10. Experimental and results

In the following, we will relate the first data we have obtained when trying to identify the conditions of separation and determination of sugars present in beet and cane molasses. Due to the complexity and the peculiar concentration ratios concerning the various sugars present in such matrixes, it would be very difficult to identify separation conditions which can allow the simultaneous quantitative evaluation of all the sugars. Therefore, as well as the qualitative separation of the various sugars, we will discuss the possibility of quantitatively determining oligosaccharides by the AMD technique and mono-saccharides by the OPLC technique.

10.1. Type of plates

In order to evaluate the ideal selectivity for the separation and quantitative determination of the sugars present in complex mixture such as molasses, we studied HPTLC plates of both the amino and diol Types (Merck, Darmstadt, Germany). Each of the two types of plates showed interesting properties, so that we can say that both of them have complementary use. In particular, we have used plates of both the amino and diol type for AMD elutions and plates of the amino type with OPLC developments. The amino plates show better selectivity for the separation of mono- and oligo-saccharides in products of sugar manufacture, but they must be previously buffered to avoid the reaction of the amino group of the phase with the reducing sugars which may be present. The latter operation can be carried out by dipping the plate into an aqueous solution of monopotassic phosphate (0.4 M) for 15 min so that the pH of the amino phase changes from 9 to about 6.5. Such conditions are

Table 2
Conditions for the separation of complex mixtures of sugars on two different types of plates

Plate:	Amino	Diol
Gradient: (acetonitrile–acetone (1:1)–water	Linear decrease of water% from 45% to 20% — 15 steps	Linear decrease of water% from 15% to 6% — 7 steps
Derivatization:	α-Naphthol (Anderton et al., 1993) direct reaction in situ* (Klaus et al., 1989)	4-Aminobenzoic acid (Jork et al., 1990)
Detection: absorbance U.V.	$\lambda_{max} = 515$ nm *$\lambda_{excitation} = 365$ nm (Hg) cut-off filter = 400 nm	$\lambda_{max} = 400$ nm $\lambda_{excitation} = 365$ nm (Hg) cut-off filter = 400 nm
Limit of detection:	20–30 ng	3–10 ng
Total time of AMD development:	4 h	2 h

unfavourable for the formation of glycosylamines (Doner and Biller, 1984). Such a treatment presents a further advantage in that it allows a higher 'sample capacity' in comparison with nonderivatized plates, whilst maintaining the property of holding (near the starting line) those nonsugars which could interfere in the colour reactions of the analytes of interest.

10.2. Standards

As oligosaccharides standards we used, as well as raffinose, a mixture of fructooligosaccharides present in the commercial product Actilight P® which is manufactured by Beghin-Meiji Industries, Paris, (which contains 35% of 1-kestose, 50% of nystose and 10% of fructosyl-nystose) and a mixture of kestose isomers obtained from cane sugar and supplied by the Sugar Milling Research Institute, Durban, South Africa; this latter mixture contains 16.5% of 1-kestose, 44.2% of neo-kestose and 10.9% of 6-kestose. After some preliminary tests were carried out on various mixtures of such products with glucose, fructose, sucrose and raffinose, we were able to identify the exact location of the various sugars on the plates depending upon the different elution conditions.

11. AMD

11.1. Gradient conditions

Bearing in mind that the AMD technique can reveal, because of the mechanism of spot reconcentration, components which are present in low concentrations, it was utilized for the separation of the oligosaccharides present in molasses. We have utilized HPTLC aminopropyl and diol plates and, as the eluent, mixtures of acetonitrile–acetone (1:1)–water. The optimum conditions for the separation and identification of the various oligosaccharides have been normalized. In Table 2 the optimum operational parameters for the two types of plates are summarized.

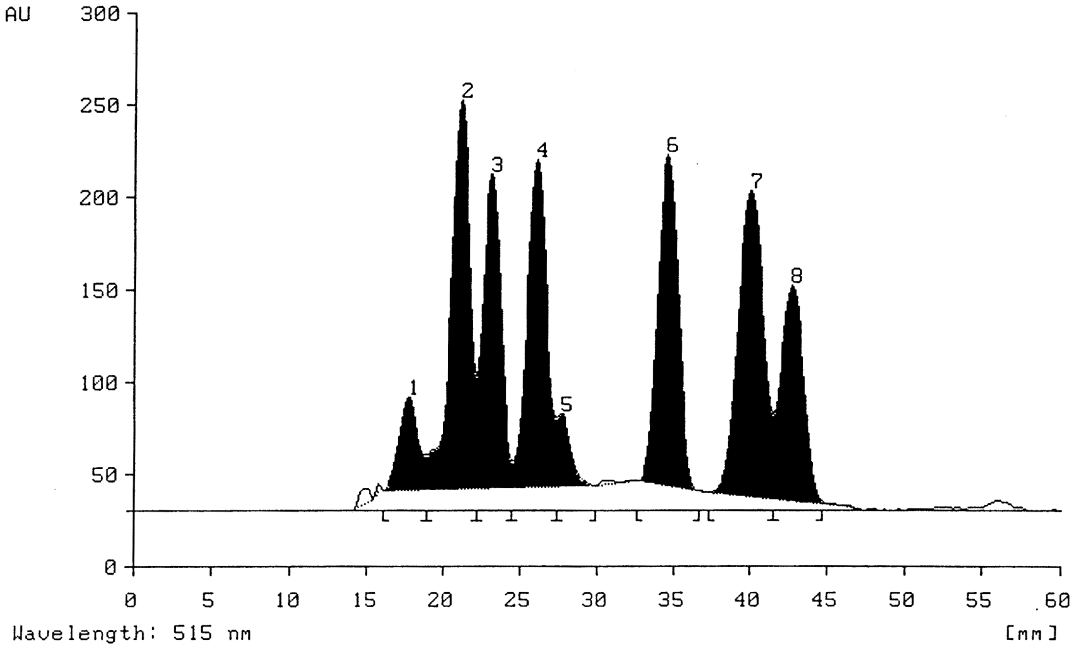

Fig. 6. Separation of standard mixture of sugars on amino plate: (1) fructosyl-nystose; (2) nystose; (3) raffinose + 6-kestose; (4) 1-kestose; (5) neo-kestose; (6) sucrose; (7) glucose; and (8) fructose.

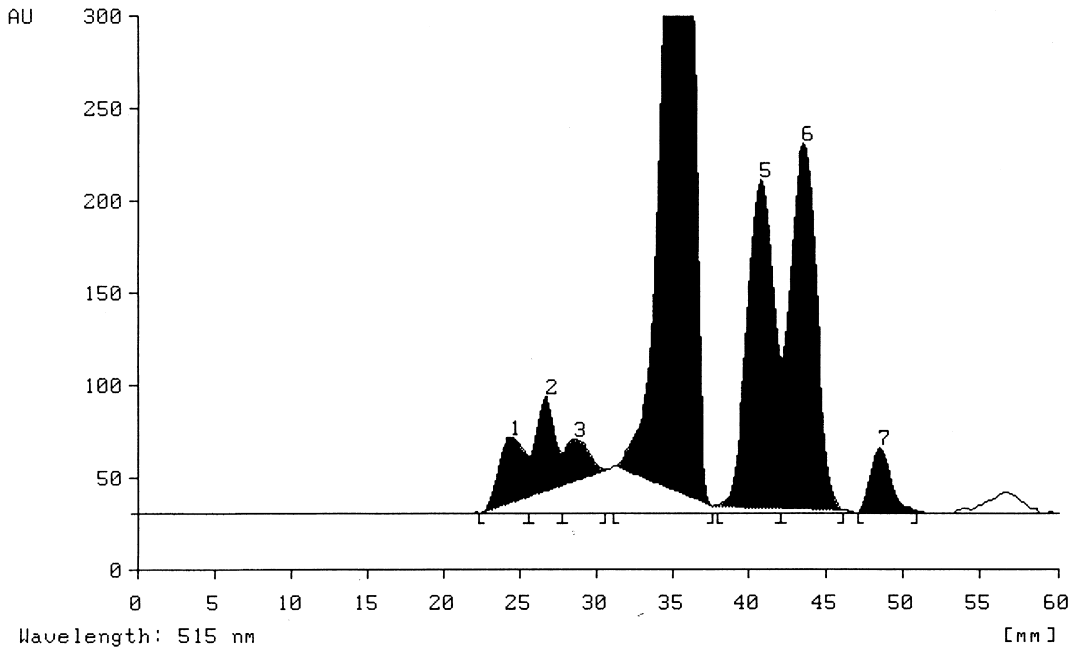

Fig. 7. Densitometric read-out of a cane molasses sample: (1) raffinose + 6-kestose; (2) 1-kestose; (3) neo-kestose; (4) sucrose; (5) glucose; (6) fructose; and (7) unknown.

11.2. Amino plates

Fig. 6 shows the densitometric read-out for the separation of a standard mixture of sugars. It can be seen that raffinose and 6-kestose have the same migration distance, so that they can only be evaluated together. Preliminary calibration tests demonstrated that the correlation coefficients for the various sugars is always higher that 0.99 with a calibration range till 100–200 ng depending upon the different type of detection. The standard error for the quantitative determination varies from 1% for the major components and up to 10% for those present at lower concentrations. Cane and beet molasses have been analyzed using aqueous solutions containing 0.5% molasses; after dilution of this solution 1:1 with acetone, 2 μl are placed on the plate. Quantitative analyses have been carried out using standards solutions (20–40 ppm) of the various sugars to be determined. Figs. 7 and 8 show densitometric recordings from cane and beet molasses. The data concerning the quantitative evaluations of the oligosaccharides (g 100 g^{-1} molasses) are shown in

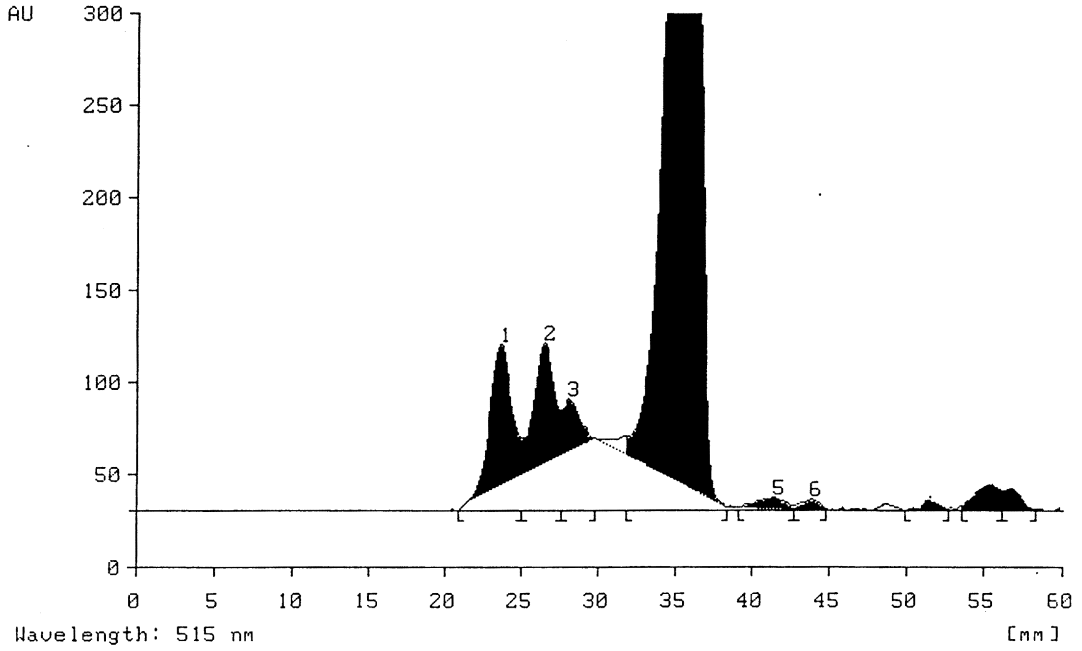

Fig. 8. Densitometric read-out of a beet molasses sample: (1) raffinose + 6-kestose; (2) 1-kestose; (3) neo-kestose; (4) sucrose; (5) glucose; and (6) Fructose.

Table 3
Oligosaccharides in cane and beet molasses (g 100 g^{-1} molasses); standard deviations in brackets

Molasses	Raffinose + 6-kestose	1-Kestose	Neo-kestose
Cane	0.45(0.01)	0.40(0.02)	0.20(0.02)
Beet	1.22(0.04)	0.45(0.01)	0.15(0.01)

Table 3 where the figures in brackets are the standard deviations.

11.3. Diol plates

A densitometric reading-out of the separation of a standard mixture of oligosaccharides is shown in Fig. 9. It can be seen that 6-kestose can be separated from raffinose. However, it has the same migration distance (R_f-values) of 1-kestose, so that these two oligosaccharides can be determined as a total. Both the calibration tests and the standard errors for the various sugars have shown data analogous to those described earlier for the amino plates. Bearing in mind that the limit of detection shown by these types of plates are lower than those of the amino plates (Table 2), the molasses solution utilized for placing the spots on the plate must be more diluted. We used 2 μl of a solution obtained from 10 ml of an aqueous (1%) molasses solution diluted to 100 ml with a mixture (1:1) of acetone–water. Using the spiking method test, it was shown that there was no matrix effect depending upon the concentration as is confirmed by the fact that the two calibration and spiking lines shown in Fig. 10 are parallel. The densitometric recording of a beet molasses sample is shown in Fig. 11; the concentration of raffinose and the sum 1-kestose + 6-kestose were 1.07% (S.D. = 0.02) and 0.46 (S.D. = 0.01), respectively.

By comparing the results obtained using both amino and diol plates we have deduced that it is necessary to analyze the same molasses using both amino and diol plates in order to obtain a complete screening of the composition of oligosaccharides present in cane and beet molasses. Bearing in mind that the presence of raffinose concerns beet more than cane molasses, we can conclude that diol plates are suited for the analysis of beet molasses, whereas amino plates better fit the conditions of cane molasses.

12. OPLC

We have pointed out that the AMD technique using amino or diol plates cannot guarantee a complete separation of all the oligosaccharides. The latter can be obtained by using (Vaccari and Accorsi, 1980) silica gel plates and a mixture of 1:2 water saturated *n*-butanol–ethanol. Such a mixture cannot be utilized when adopting AMD instrumentation; on the other hand the complete separation of the various oligosaccharides via capillary elution requires a very long elution time (50 h). The problem can be solved by adopting the OPLC technique which makes it possible to considerably reduce the elution time. Our experiments in this area are still on-going and the results are still being followed up but we can give the analytical conditions for the determination of glucose and fructose in cane molasses. In fact the OPLC technique is very suitable for the analysis of components present at high concentrations in cane molasses. By using as the eluent a mixture of acetonitrile–acetone (1:1) 70/water 30, together with

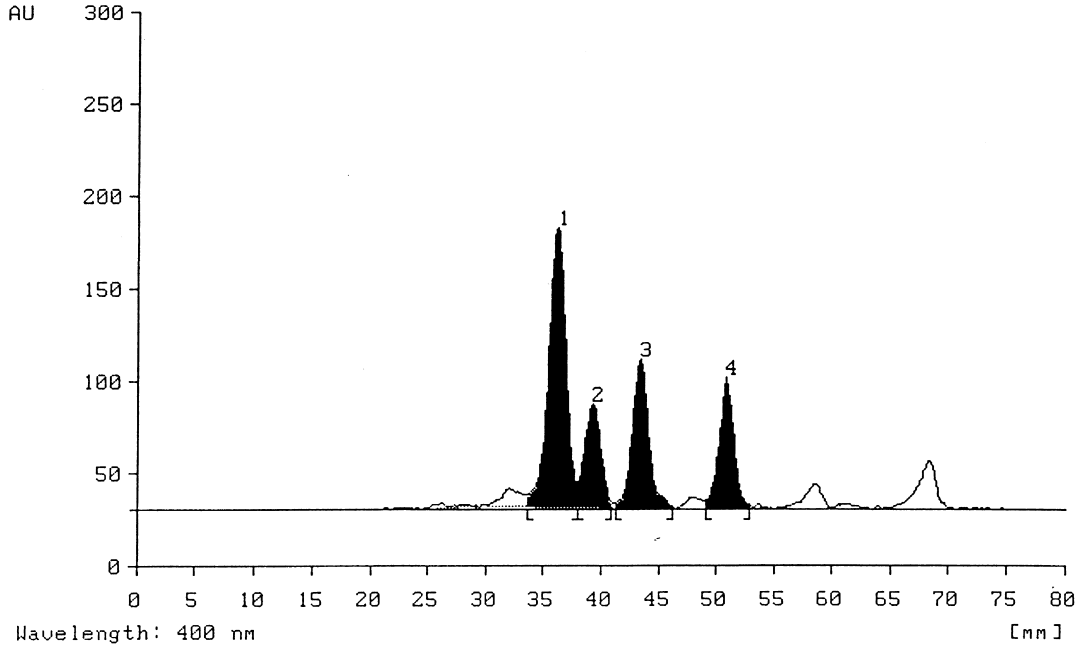

Fig. 9. Separation of oligosaccharides standards on diol plates: (1) raffinose; (2) 1-kestose + 6-kestose; (3) neo-kestose; and (4) sucrose.

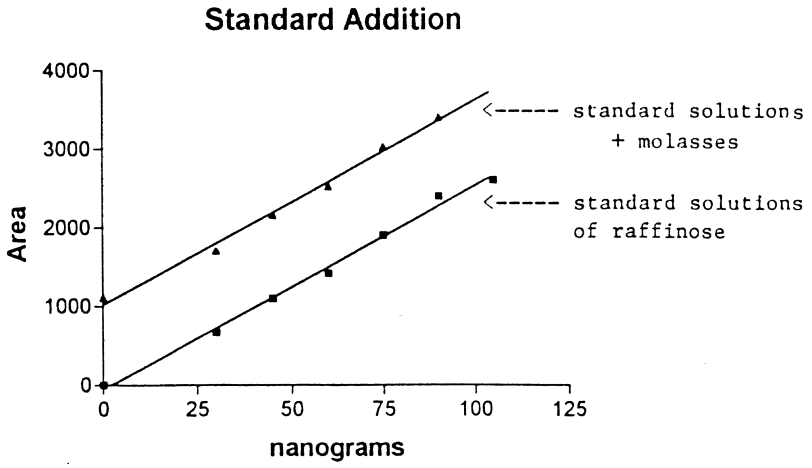

Fig. 10. Check of the matrix effect depending upon the concentration via the addition of the standard to the sample (spiking test).

24 bar pillow pressure, a 5 bar eluent pressure and 2.5 ml min^{-1} eluent flow, a 14 cm development distance could be covered within 18 min. Such conditions allow an optimum separation of glucose and fructose, the calibration curve of which (correlation coefficient > 0.99) extends down to 200 ng. Using such a technique we were able to obtain good results in the direct analysis of cane molasses after simple dilution with water (0.5 g l^{-1}) and by placing 2 μl on the plate. Through the densitogram shown in Fig. 12 it was possible to establish that the sample of cane molasses contained 12.87% fructose (S.D. = 0.32) and 11.27% glucose (S.D. = 0.34). The sucrose and oligosaccharides contents could not be determined on the same plate due to their too high and too low concentration, respectively.

13. Conclusions

Based on our experimental results, we can claim that planar chromatography, and in particular the AMD and OPLC techniques, can be considered as successful alternatives to the traditional methods of analysis. In particular, any preliminary treatment or purification of the sample are unnecessary and a simple dilution with water is sufficient. A number of samples can be analyzed simultaneously on the same plate thus very much decreasing the specific analysis time. All the various analytical phases are completely automated and do not need highly skilled operators. Apart from the cost of the equipment, which is on the other hand relatively low, the specific cost for the analysis of a sample

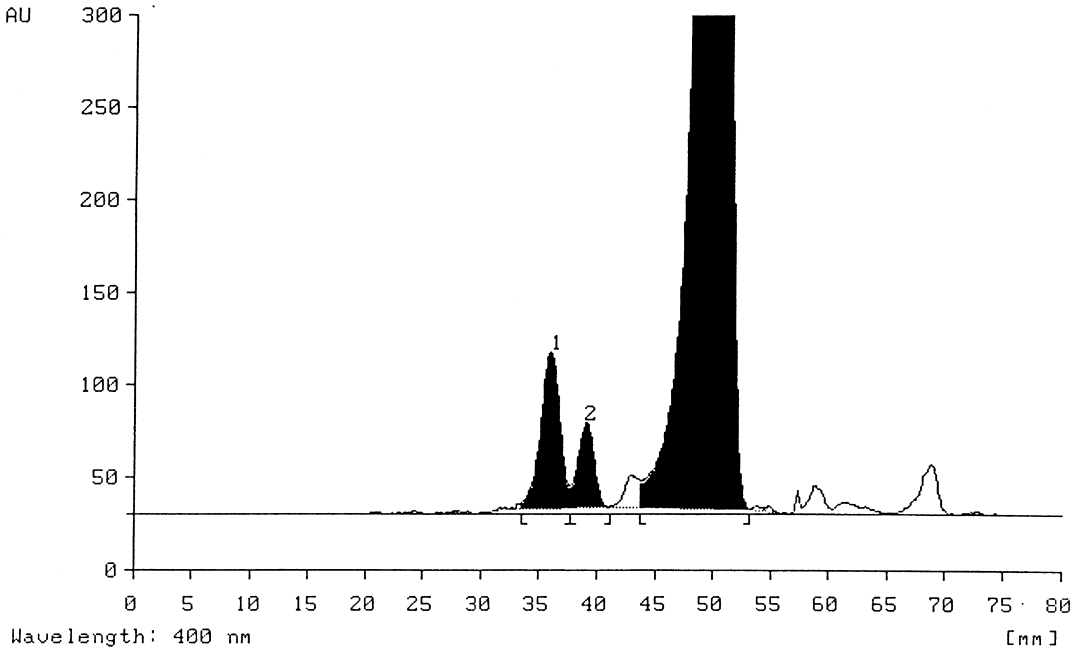

Fig. 11. Beet molasses analysis: (1) raffinose; and (2) 1-kestose + 6-kestose.

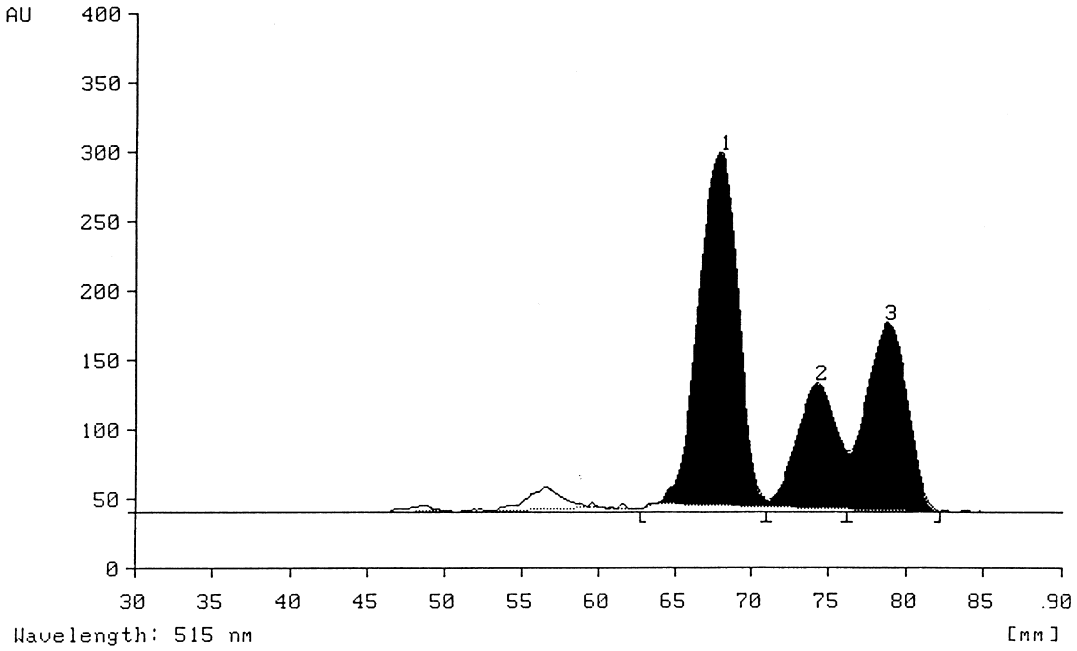

Fig. 12. Analysis of cane molasses: (1) sucrose; (2) glucose; and (3) fructose.

is quite small. Even the specific time for the analysis of a sample is very cheap bearing in mind that a number of samples can be analyzed on the same plate. Moreover, the OPLC technique is very rapid and the AMD elution can be carried out overnight. Finally, if we remember that many components present in the same sample can be determined simultaneously and, as well as molasses, other sugar factory juices and raw sugars can be analyzed, we can conclude that such an analytical technique fits many of the requirements mentioned in the preamble. However, the whole sugars content cannot be determined on the same plate and under the same operation conditions; for example, for the separation of different sugars co-eluted, the type of layer or the elution conditions must be modified.

References

Anderton, C. A., Fried, B., & Sherma, J. (1993). HPTLC determination of sugars in the hemolymph and digestive gland–gonad complex of biomphalaria glabrata. *J. Planar Chromatogr., 5*, 51–54.

Doner, L. W., & Biller, L. M. (1984). High performance thin-layer chromatographic separation of sugars: preparation and application of aminopropil bonded-phase silica plates impregnated with monosodium phosphate. *J. Chromatogr., 287*, 391–398.

Goulding, R. W. (1975). Liquid chromatography of sugars and related polyhydric alcohols on cationexchangers. *J. Chromatogr., 103*, 229–239.

ICUMSA — Proceedings of 20th Session — Subject 19: Oligosaccharides and Polysaccharides Colorado Spring, USA, 1990, pp. 358–380. ICUMSA, Peterborough, England.

Klaus, R., Fisher, W., & Hauck, H. E. (1989). Use of a new adsorbent in the separation and detection of glucose and fructose by HPTLC. *Chromatographia, 28*, 364–366.

Jork H., Funk W., Fisher W., & Wimmer H. (1990). *Thin Layer Chromatography*. Weinheim, Germany: VCH, pp. 154–156.

Linden, J. C., & Lawhead, C. (1975). Liquid chromatography of saccharides. *J. Chromatogr., 105*, 125–133.

Partridge A. (1965). In E. Stahl, & U. Kaltenbach, *Thin Layer Chromatography*. Berlin: Springer, p. 461.

Schneider F. (Ed.) (1979). *Sugar Analysis — ICUMSA METHODS.*

Stahl, E., & Kaltenbach, U. (1961). Mitteilung, spurenanalyse von zuckergemischen auf kieselgur Gschichten. *J. Chromatogr., 5*, 351–355.

Tyihak, E., & Minczovics, E. (1979). New planar liquid chromatography technique: overpressured thin-layer chromatography. *J. Chromatogr., 174*, 75–81.

Vaccari, G., & Accorsi, C.A. (1980). L'applicazione dell'analisi cromatografica in zuccherificio. *Ind. Sacc. Ital., 78*, 121–133.