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Chromatographic Separations in Sugar Analysis and Processes

Detection of oligosaccharides in sugar products using planar chromatography

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Abstract

Oligosaccharides, and in particular raffinose and kestoses, are of great importance not only in the field of cane and beet processing but also in respect of the analyses of a number of agricultural raw materials and trade products. The authors judge it to be useful to have at one's disposal a simple and reliable analytical technique to be used for the detection of oligosaccharides in different materials. Modern planar chromatography can be utilized, bearing in mind that HPTLC (high performance thin layer chromatography) plates, Automated Multiple Development, completely automated elution systems, sample positioning and spots detection apparatuses, are available. Details on the analytical methodology adopted for molasses and other sugar products are presented and discussed also giving statistical data about its accuracy and precision. Some examples of practical application of the proposed methodology are described.

Keywords: Planar chromatography; Oligosaccharides

1. Introduction

The analytical and technological problems related to the presence of oligosaccharides in beet and cane processing are well known. In particular, the amount of raffinose leads to a falsely high polarization, it slows down the kinetics of crystal growth and it causes the formation of sucrose needle crystals which are difficult to centrifuge and pack (Vaccari, Mantovani, Sgualdino, Aquilano, & Rubbo, 1986). Recently it has been pointed out that kestoses can also promote major habit modification of sucrose crystals due to their presence inside the lattice which causes a distortion of the lattice itself (Vaccari, Mantovani, Sgualdino, Tamburini, & Aquilano, 1999; Vaccari, Sgualdino, Tamburini, Lodi, Aquilano, & Mantovani, 1999).

Other than the problems related to beet and cane processing, oligosaccharides, and kestoses in particular, are becoming increasingly important due to their characteristics as pre-biotic compounds (Sungsoo Cho, Prosky, & Dreher, 1999). The commercial production of mixtures of kestoses, obtained via enzymatic processes, is in operation industrially (Quarne & Guibert, 1996) but extraction processes from different raw materials are being studied; moreover transgenic sugar beet is being investigated with the objective of obtaining a crop that produces fructo-oligosaccharides (Sévenier, Hall, van der Meer, Hakkert, van Tunen, & Koops, 1998).

It is clear from the points mentioned above how important the setting up of simple and reliable analytical techniques can be for the detection of oligosaccharides in different materials. We believe that modern planar chromatography can be utilized, bearing in mind that HPTLC plates and Automated Multiple Development (AMD) completely automated elution systems, including sample positioning and spots detection, are available. Such a methodology satisfy the following requirements:

1. simple pre-treatment;
2. automation of the various steps;
3. simultaneous analysis of a number of samples;
4. simultaneous evaluation of a number of analytical parameters;
5. limited cost for each sample analysis;
6. possibility of analysis of samples of very different molecular structure and polarity; and
7. high sensititivity and good reproducibility.

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The purpose of this paper is to propose a routine method to be utilized not only in the sugar industry but also for the analysis of various products other than in the research field. We would like to stress some specific applications of the proposed technique and its usefulness for the analysis of different products.

2. Methodology

The present application utilises modern instrumental thin layer chromatography, which meets most of the criteria mentioned above (Mantovani, Vaccari, Dosi, & Lodi, 1998). and in particular gives a rapid method for the detection and quantitative determination of the oligosaccharides in beet molasses and other products. AMD (Burger & Tengler, 1986) is employed because it allows reproducible separation of very different compounds in complex mixtures. The unquestionable advantage of gradient AMD for the separation of complex saccharides mixture is shown in Fig. 1 as a typical example.

In the present application Diol layers are chosen as the stationary phase because:

1. Diol is selective enough to separate many oligosaccharides (Lodi, Bighi, Brandolini, Menziani, & Tosi, 1997) although 1-kestose and 6-kestose are not separated. The utilization of amino layers could separate 1-kestose from 6-kestose but raffinose and 6-kestose are then eluted together (Mantovani et al., 1998).
2. Diol requires less water with respect to the more polar amino and silica layers for development with aqueous eluents.

Unfortunately, among the three kestoses (1-, 6- and *neo*-kestose) only 1-kestose is available as a pure standard. Hopefully the other isomers will be available in the near future. Meanwhile, assuming that the response factors of the lacking kestoses are not very different from that of 1-kestose, it is possible to gain quantitative information about them from the 1-kestose calibration curves.

2.1. Solvents and standards

Acetone and Acetonitrile were of HPLC quality (Becker, Deventer, Holland). Water was ultrapure. Raffinose hydrate (lot 38056; 99.6%; water 15.0%) was

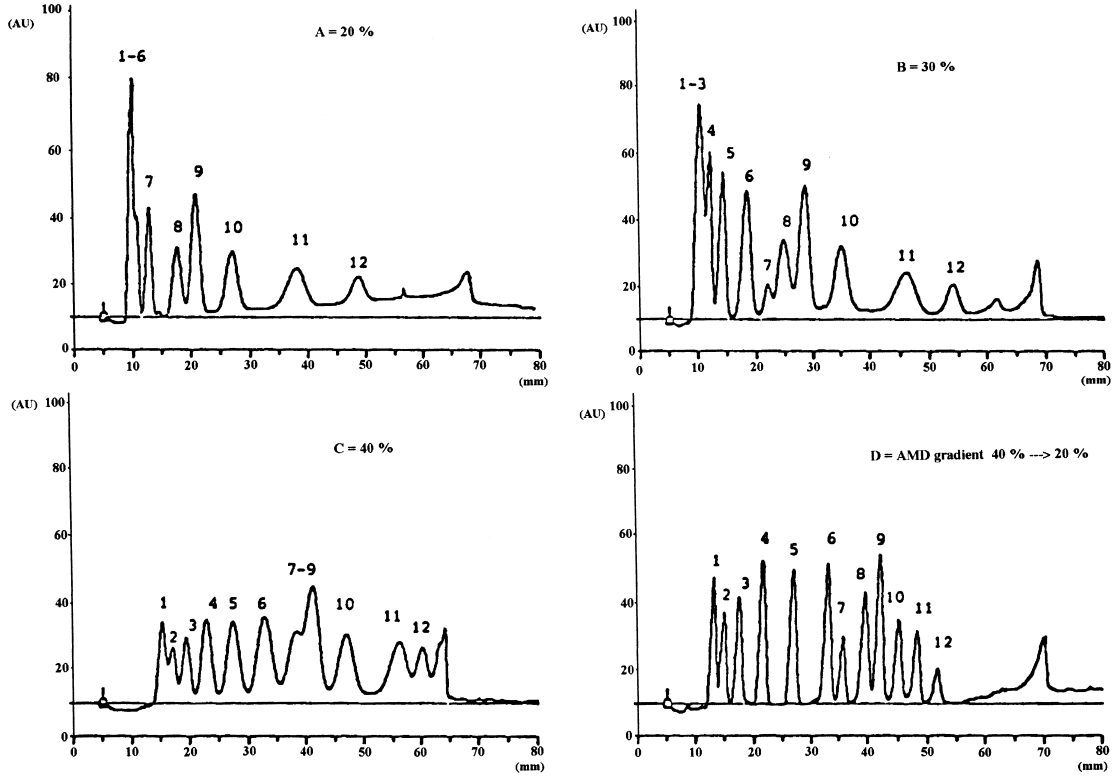


Fig. 1. Densitogram curve of a mixture of 12 saccharides with very different molecular structures: (1) maltoheptose; (2) maltohexose; (3) maltopentose; (4) maltotetrose; (5) maltotriose; (6) maltose; (7) sucrose; (8) glucose; (9) fructose; (10) xylose; (11) rhamnose; (12) desoxy-ribose. Layer Amino (Merck); (A) isocratic development acetonitrile:acetone (1:1)/water (80 + 20); (B) isocratic development acetonitrile:acetone (1:1)/water (70 + 30); (C) isocratic development acetonitrile:acetone (1:1)/water (60 + 40); (D) AMD linear gradient with water 40%→20%. Detection: fluorescence with Hg lamp at 366/ > 400 after derivatisation in situ at 150°C on plate heater (Lodi, Betti, Brandolini, Menziani, & Tosi, 1994).

BDH Chemicals Ltd (Poole, England). 1-Kestose (99.0%; water 5.0%); nystose (99.0%; water 5.0%) and fructosyl-nystose (98.0%; water 5.0%) were Wako Chemicals (Richmond, USA), lot ACG7359. 6-Kestose and *neo*-kestose were a gift from the Sugar Milling Research Institute (Durban, South Africa). Sucrose was from Eridania (Genova, Italy; 99.9%; water 0.01%). Standards were dissolved in water + acetone (2 + 1) to form 5–10 ng/μL solutions.

2.2. Preparation of samples

For the analyses of molasses, 1 g of the product was dissolved in 400 ml of distilled water. No clean-up was performed. Other kind of substances were suitably diluted without any kind of cleaning-up.

2.3. Samples and standard application

Sample application was performed with a Linomat IV (Camag); 2–13 μl of standards were applied 8 mm from the bottom of the plate as bands of 4–6 mm; the delivery rate was 15 s μl^{-1}.

2.4. Chromatography

Diol HPTLC plates 20×10 cm used for this application were from Merck (code 1.05636.0001). A nine-step gradient was performed using a Camag AMD apparatus following the conditions described in Table 1. The mixing of the solutions are, of course, completely automated in the Camag AMD apparatus. The gradient shape is shown in Fig. 2. Development times (min) for the various steps are, respectively: 1, 1.8, 2.8, 4.2, 6.3, 8.5, 11.5, 14.5, and 18.

2.5. Derivatization

Derivatization was performed with 4-aminobenzoic acid reagent (Jork, Funk, Fisher, & Wimmer, 1990): glacial acetic acid (36 ml), water (40 ml), 85% phosphoric acid (2 ml) and acetone (120 ml) added to 4-aminobenzoic acid (2 g). This acetonic solution can be kept for 2 weeks at −18°C with no loss in its reactivity.

The developed plates were dipped into the reagent by means of a Camag dipping apparatus set at an immersion rate of 1 s and immersion time of 2 s. The dipped layers were laid down to dry then heated in a oven at 115°C (15 min). After heating, yellowish to brown spots on a pale yellow background appear. The derivatized plate shows no marked modifications if it is kept tightly sealed with a glass cover at −18°C.

Table 1
Elutions conditions using AMD apparatus

Step	Solution 1[a]	Solution 2[b]	Solution 3[c]
1	100		
2	81.38	18.62	
3	52.59	47.41	
4	33.98	66.02	
5	21.96	78.04	
6	14.19	67.19	18.62
7	9.17	43.42	47.41
8	5.92	28.06	66.02
9	3.82	18.13	78.04

[a] Acetonitrile/acetone (1:1 v/v)/water = 85/15
[b] Acetonitrile/acetone (1:1 v/v)/Water = 94/6
[c] Acetonitrile/acetone (1:1 v/v)/Water = 95/5

2.6. Scanning

The derivatized layers were scanned with a Camag Scanner III interfaced with a computer and Cats 4.03 evaluation software, in both fluorescence with Hg lamp 366/ > 400 and absorbance mode ($\lambda_{max} = 400$ nm).

2.7. Results

Fig. 3 shows the separation of the oligosaccharides standards obtained with the AMD gradient described above. Fig. 4 shows the increase of information obtained when using AMD gradient vs. isocratic development with a sample of molasses. Calibration curves of raffinose and fructo-oligosaccharides were investigated in the range from low nanograms to about 300 ng/spot. Peak heights detected both in fluorescence and absorbance fit a polynomial of the second order. Pseudo-linear ranges of 0–130 ng/spot were found for absorbance (Fig. 5a) while for fluorescence (Fig. 5b) the linear range was further restricted. In the following we report some practical applications of the described methodology.

3. Applications

3.1. Quantitative determination of raffinose and kestoses in molasses from different countries

Screening of molasses from several countries showed no detectable amounts of nystose and fructosyl-nystose and very different concentrations of raffinose and 1 + 6 kestoses, while *neo*-kestose was present mostly at trace levels.

For the present application a general method for the screening of any unknown molasses has been devised:

1. prepare a standard solution containing both 1-kestose (at 5 ng/μL) and raffinose (at 10 ng/μL);
2. carry out the deposition of 2, 4, 6, 8, 10 and 13 μl of the above standard solution at 1–6 and 4 μl of 8 different samples at a–h according to the following scheme: a-1-b-2-c-d-3-e-4-f-5-g-6-h.;

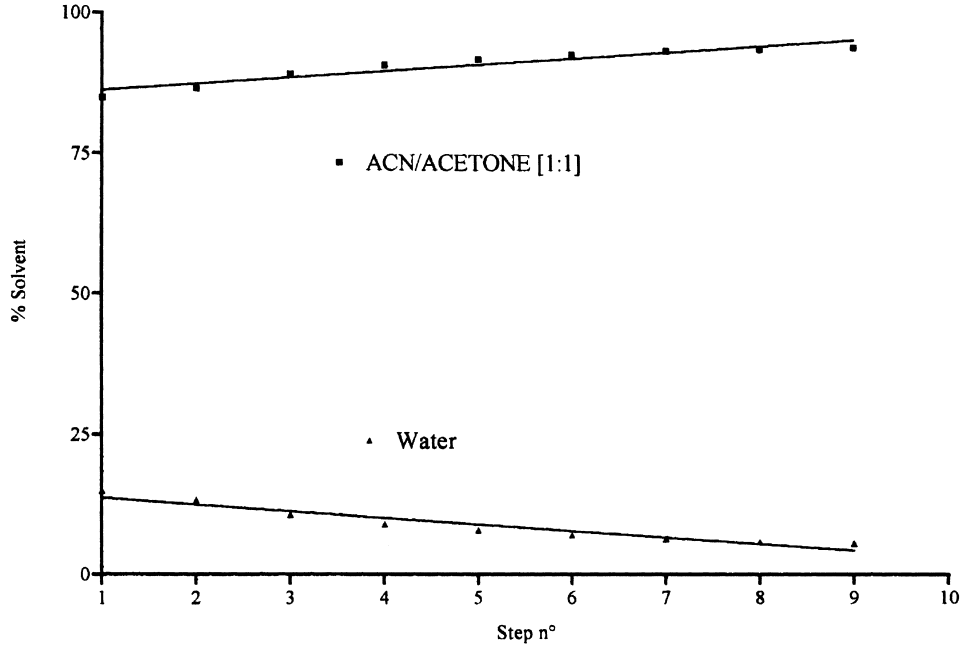


Fig. 2. Profile of nine-step AMD linear gradient optimized for the separation of oligosaccharides in beet molasses.

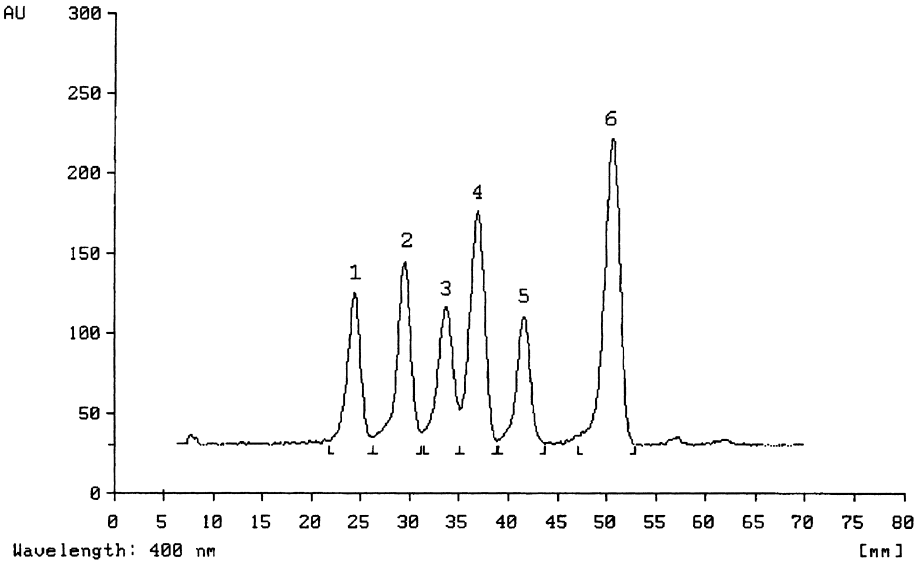


Fig. 3. Separation of: (1) fructosyl-nystose 90 ng; (2) nystose 120 ng; (3) raffinose 75 ng; (4) 1-kestose + 6-kestose (combined) 140 ng; (5) *neo*-kestose 70 ng; (6) sucrose 200 ng.

3. run the AMD gradient;
4. carry out the visualization as described above in Section 2.5 and the densitometry in absorbance mode at $\lambda_{max} = 400$ nm (W);
5. information about the concentration of raffinose and 1 + 6 kestoses in each of the samples under investigation (Fig. 6) is easily obtained from the Cats software; and
6. information about the neo-kestose concentration can be obtained from the calibration curve of the 1-kestose.

On the basis of the preliminary results (Fig. 6), quantitative determination of raffinose and 1 + 6 kestoses can be carried out using the same solution of the standards used for the screening in the same calibration range, i.e. 20–130 ng/spot for raffinose and 10–65 ng/spot for the 1-kestose and the same solution (1:400) of molasses samples. However, one has to check which volume has to be deposited for each sample in order to adequately interpolate in the calibration curve. For example (Table 2) it is possible to carry out the quantitative analysis of the molasses from (1) England, (2) Germany and (3) Austria

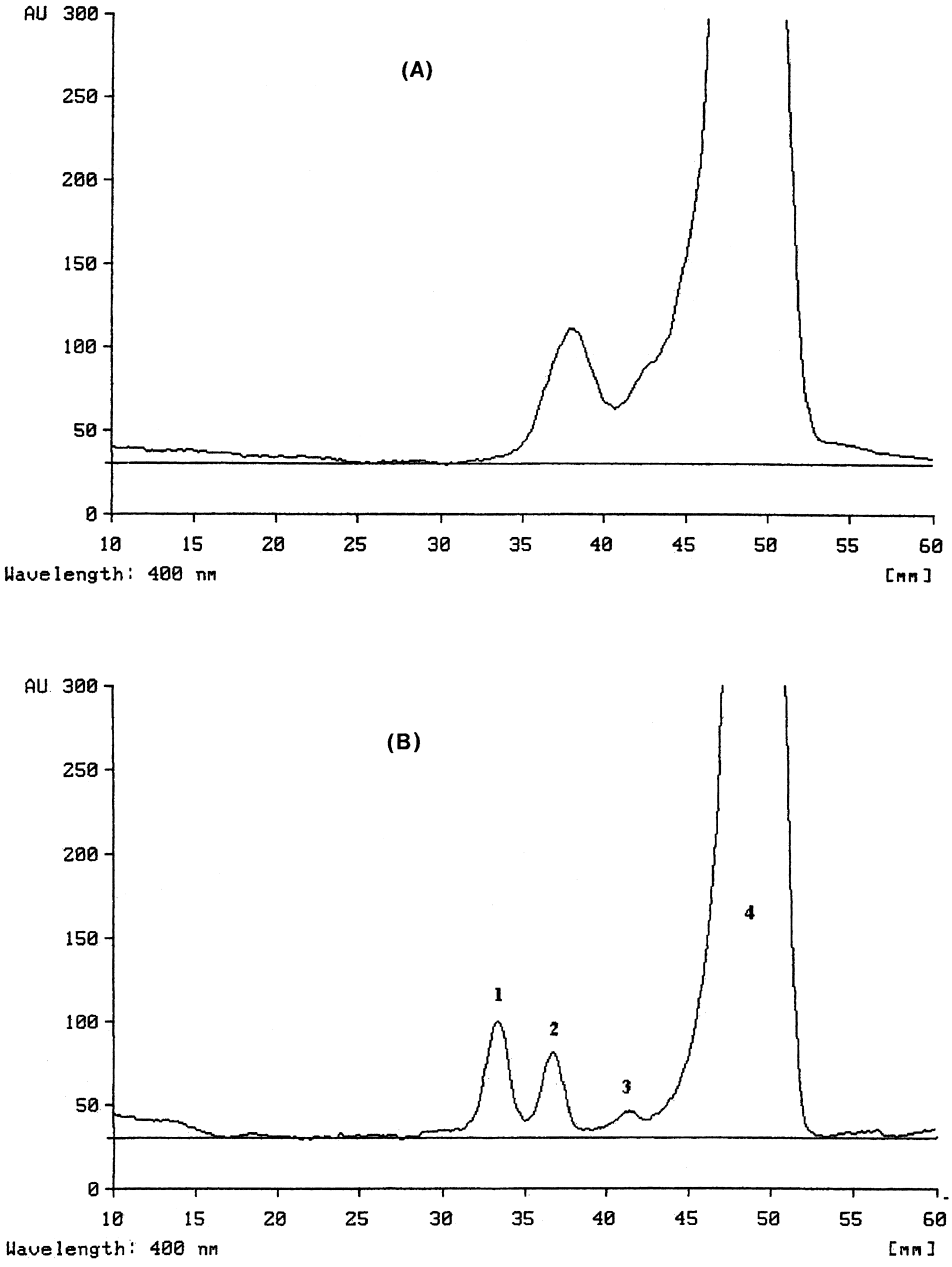


Fig. 4. Comparison between (A) isocratic and (B) gradient development for the separation of the oligosaccharides in a beet molasses: (1) raffinose; (2) 1-kestose + 6-kestose; (3) *neo*-kestose; (4) sucrose.

on the same plate with three replicates of 4 μL of each using the following scheme: 1-a-b-2-c-3-a-b-4-c-5-a-b-6-c. In this way it is possible to determine both raffinose and 1 + 6-kestoses with a single run. The results reported in Table 2 have been obtained following this criterion with 2 or 3 samples/plate. Of course, fine tuning for each analyte can be done using single level calibration.

Although a comparison among the composition of the different molasses (Table 2) can be very risky, we can observe that the greatest amount of raffinose, as expected, is found in Northern European molasses whilst the greatest amount of kestoses is found in molasses produced in the Southern latitudes.

3.2. Investigation on the morphological effects of kestoses on sucrose crystals

Experiments recently carried out on the direct raw juice crystallization in the absense of the traditional calco-carbonic purification (Vaccari, Mantovani, Sgualdino, Tamburini, & Dosi, 1999a) demonstrated out that sucrose crystals obtained presented a peculiar morphology.

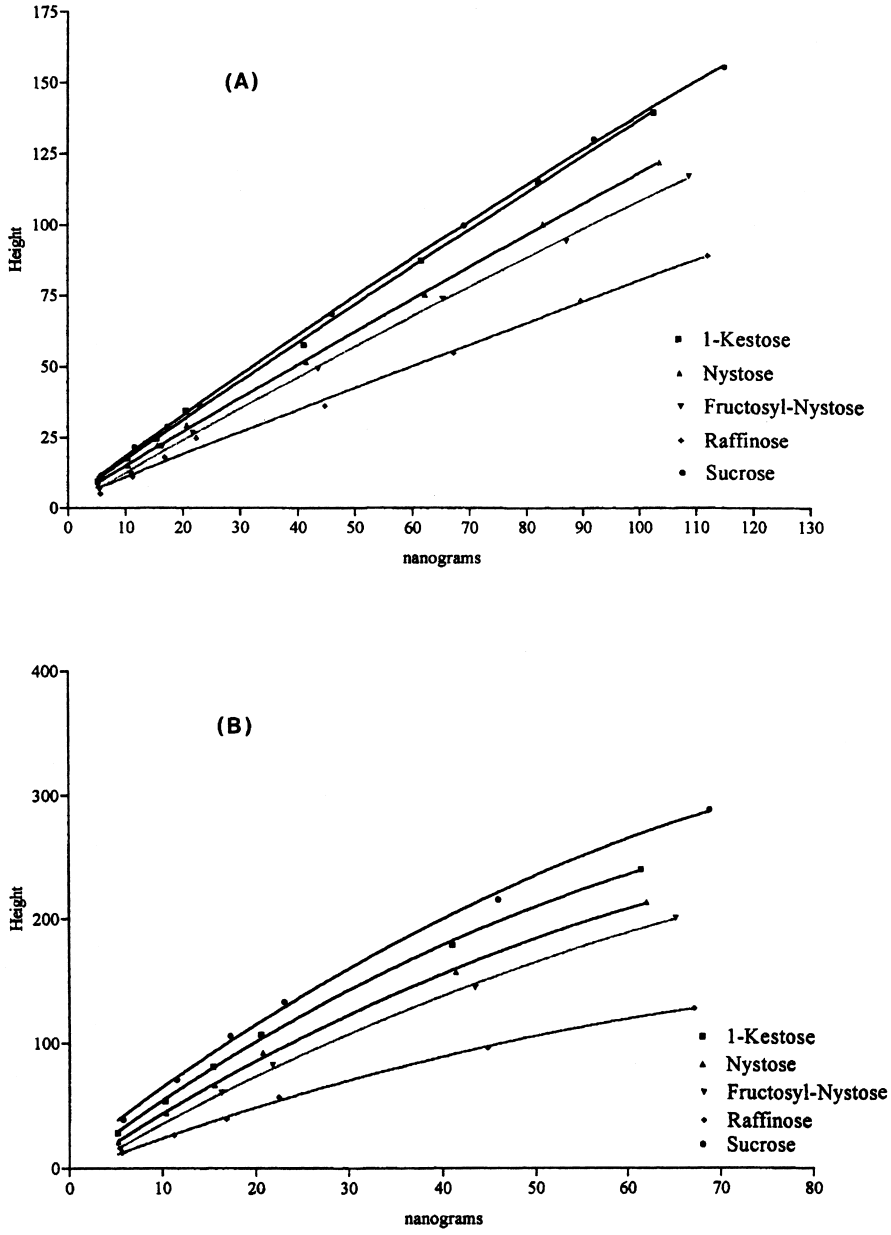


Fig. 5. Calibration curves of the five commercially available oligosaccarides, relevant in beet molasses. In the absorbance mode (A) the range of linearity spans about 0-130 ng/spot; in fluorescence mode the sensitivity is greatly increased, however, the linearity range is restricted.

Table 2
Oligosaccharides in beet molasses (g/100 g molasses; standard deviation in parenthese)

Country of origin of molasses	Raffinose	1 + 6 Kestoses	*Neo*-kestose
The Netherlands	0.72 (0.03)	0.05 (0.01)	–
England	0.85 (0.02)	0.33 (0.01)	0.06 (0.01)
Italy	0.35 (0.01)	0.47 (0.01)	0.05 (0.01)
Germany	1.01 (0.02)	0.28 (0.01)	–
Russia	0.63 (0.04)	0.16 (0.01)	–
Turkey	0.68 (0.01)	0.43 (0.01)	0.04 (0.01)
Morocco	0.25 (0.01)	0.50 (0.03)	0.29 (0.02)
Austria	0.79 (0.02)	0.33 (0.01)	0.04 (0.01)

Preliminary experiments using planar chromatography suggested that such morphology was due to the presence of fructo-oligosaccharides in the raw juice. Consequently, we tried to study in detail the shape of sucrose crystals grown in the presence of *Actilight® P*, (a commercial product put on the market by Béghin-Meiji Industries — Paris, France) the composition of which is claimed to be as described in Table 3.

The sucrose crystals obtained exhibited a triangular morphology (Fig. 7a) which became rombic (Fig. 7b) when the crystals reached large dimensions (Vaccari, Mantovani et al., 1999).

With the aim of explaining why fructo-oligosaccharides change sucrose crystal morphology, we utilized planar chromatography and the back-developed elution technique (Vaccari, Sgualdino et al., 1999) for the analyses of the crystals. Analyses carried out either on the whole crystals or on their right and left poles, have shown that not only were the concentrations of fructo-oligosaccharides present in the two poles different (Fig. 8), but also that there was a clear presence of *neo*-kestose, the concentration of which, inside the crystals, was higher than the concentration of the other fructo-oligosaccharides.

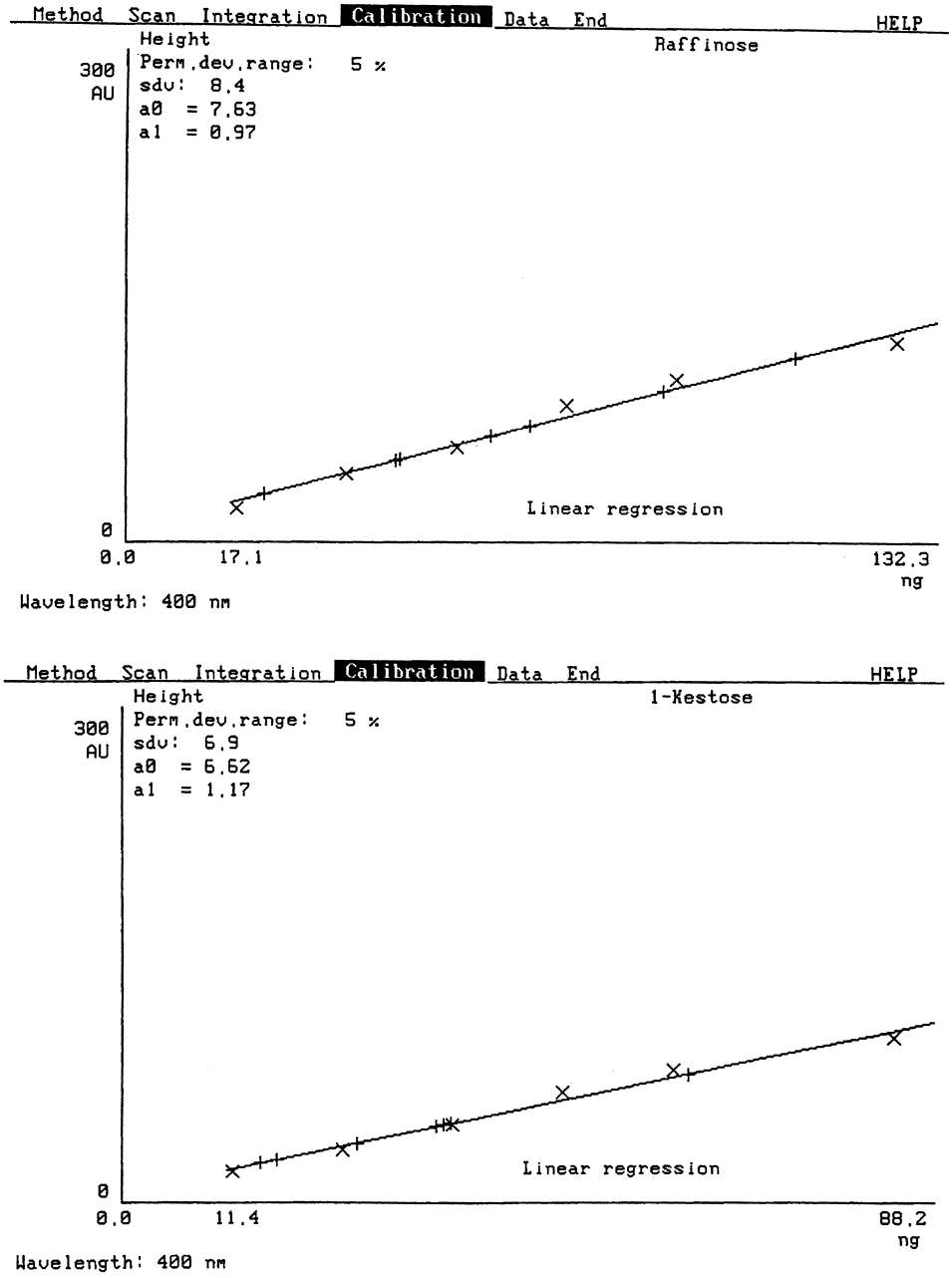
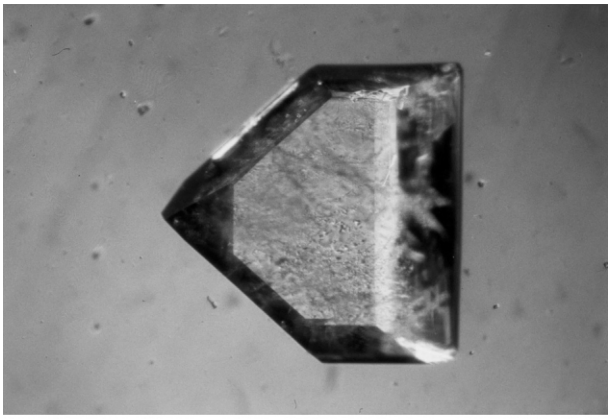


Fig. 6. Screening of the raffinose and 1-kestose + 6 kestose in eight beet molasses from different countries.

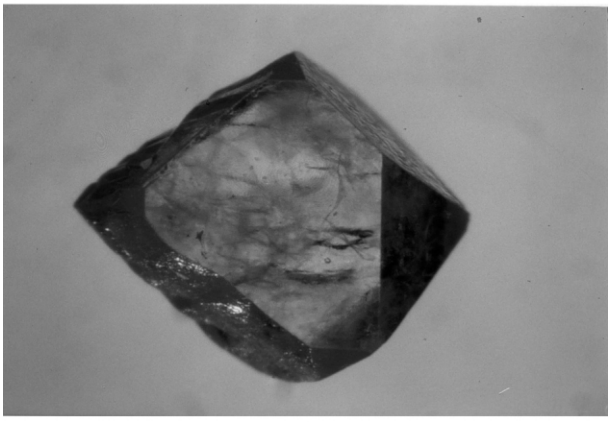
We wondered which was the origin of the *neo*-kestose. A detailed analysis of *Actilight®P* made using planar chromatography, showed that *neo*-kestose was present, although in very small concentration (≈ 3%) in the mixture of impurities we utilized. The contrast between the lowest concentration of *neo*-kestose in the growing solution and it having the highest concentration in the sucrose crystals obtained, supported the hypothesis that *neo*-kestose can markedly interfere with the crystal lattice (Vaccari, Sgualdino et al., 1999).

Table 3
Composition of *Actilight®P*

Glucose + Fructose + Sucrose	⩽ 5%
Fructo-oligosaccharides	⩾95%
1-Kestose	35±3%
Nystose	50±3%
Fructosyl-nystose	10±3%



(a)



(b)

Fig. 7. Sucrose crystals grown in the presence of *Actilight®P*.

3.3. Correlation between molasses composition and sucrose crystal morphology

In the framework of our studies on raw juice cooling crystallization (EC COPERNICUS Project ERBIC 15-CT96-0734), having the objective of eliminating the calco-carbonic purification process in the traditional beet sugar technology, we have compared the cooling crystallization of microfiltered raw juice and traditional thick juice produced in the same sugar factory in the North of France. Without describing here the details of the whole research, we would just like to emphasize that the morphology of sucrose crystals obtained from the two juices was completely different.

The analysis of the molasses obtained from the two different juices, using planar chromatography previously described showed (Fig. 9) that practically only raffinose is present in molasses originating from calco-carbonic purification juice (Table 4) whilst considerable amounts of 1-kestose and 6-kestose, as well as a small amount of *neo*-kestose, are present in molasses originating from the raw juice.

From these data we can draw the following conclusions:

1. the different morphology of crystals is due to the different amount of oligosaccharides present in the juices; and
2. most of the fructo-oligosaccharides are decomposed in the calco-carbonic purification process and practically only raffinose remains.

3.4. Correlation between the composition of the molasses obtained from cooling crystallization of the "extract" produced from chromatographic simulated moving bed (SMB) treatment of raw juice and the morphology of the sucrose crystals

Planar chromatography allowed us to study the morphology of sucrose crystals originating from the various steps of cooling crystallization of the "extract" obtained from the chromatographic treatment of beet raw juice. This research has been carried out in cooperation with the "Amalgamated Sugar Company", once again with the aim of eliminating the traditional calco-carbonic purification process. All the results of this research have been presented at PRES'2000 Congress in Prague (Vaccari, Tamburini, Sgualdino, Kearney, Kochergin, & Costesso, 2000). The planar chromatographic analysis of molasses (Fig. 10) shows that, as well as raffinose, a considerable quantity of fructo-oligosaccharides, *neo*-kestose included, are present.

From the chromatogram the following composition has been obtained: raffinose, 3.82%; 1-kestose + 6-kestose, 1.90%; and *neo*-kestose, 1.13%. From these data, and knowing the effect of poisoning of the different

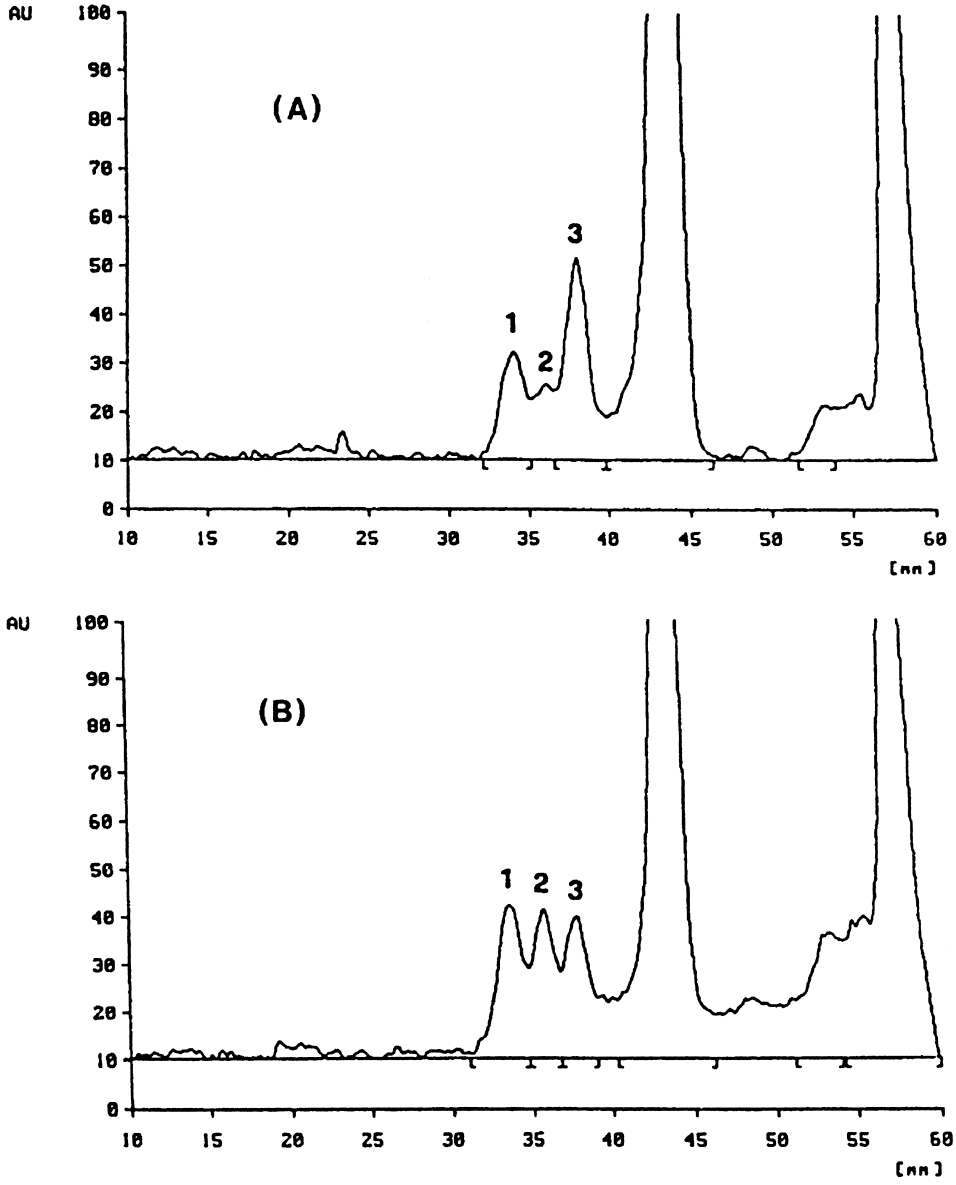


Fig. 8. Densitogram of (A) the right pole and (B) the left pole of a sucrose crystal nucleated and grown in the presence of *Actilight®P*. (1) Nystose; (2) 1-kestose; (3) *neo*-kestose.

oligosaccharides on the various faces of the sucrose crystal, we were able to jusify the peculiar morphology of the crystals obtained.

3.5. Investigation on the composition of leaves of the industrial artichoke

On the possible applications of planar chromatography for the analysis of oligosaccharides, we would like to mention its utilization in another field, not directly related to the beet, viz. the industrial utilization of artichokes. It is well known that this industry utilizes only the core of the artichoke whilst the leaves, which represent about 60% of the product, are used as fodder. It is also known that artichokes, leaves included, contain sugars ranging from monosaccharides to inulins via fructo-oligosaccharides (Bacon & Edelman, 1951). These products, such as *Actilight®P*, are becoming increasingly important from the commercial point of view due to their characteristics as pre-biotic compounds (Sungsoo et al., 1999).

We are studying the possibility of extracting this mixture of sugars, having high value-added, from the artichoke leaves before their utilization as fodder. We have obtained a white powder made up of a mixture of sugars which, via planar chromatography, gives the chromatogram shown in Fig. 11.

The presence of various fructo-oligosaccharides of different molecular weights is clear as well as the presence of simple sugars and polymers, i.e. inulins.

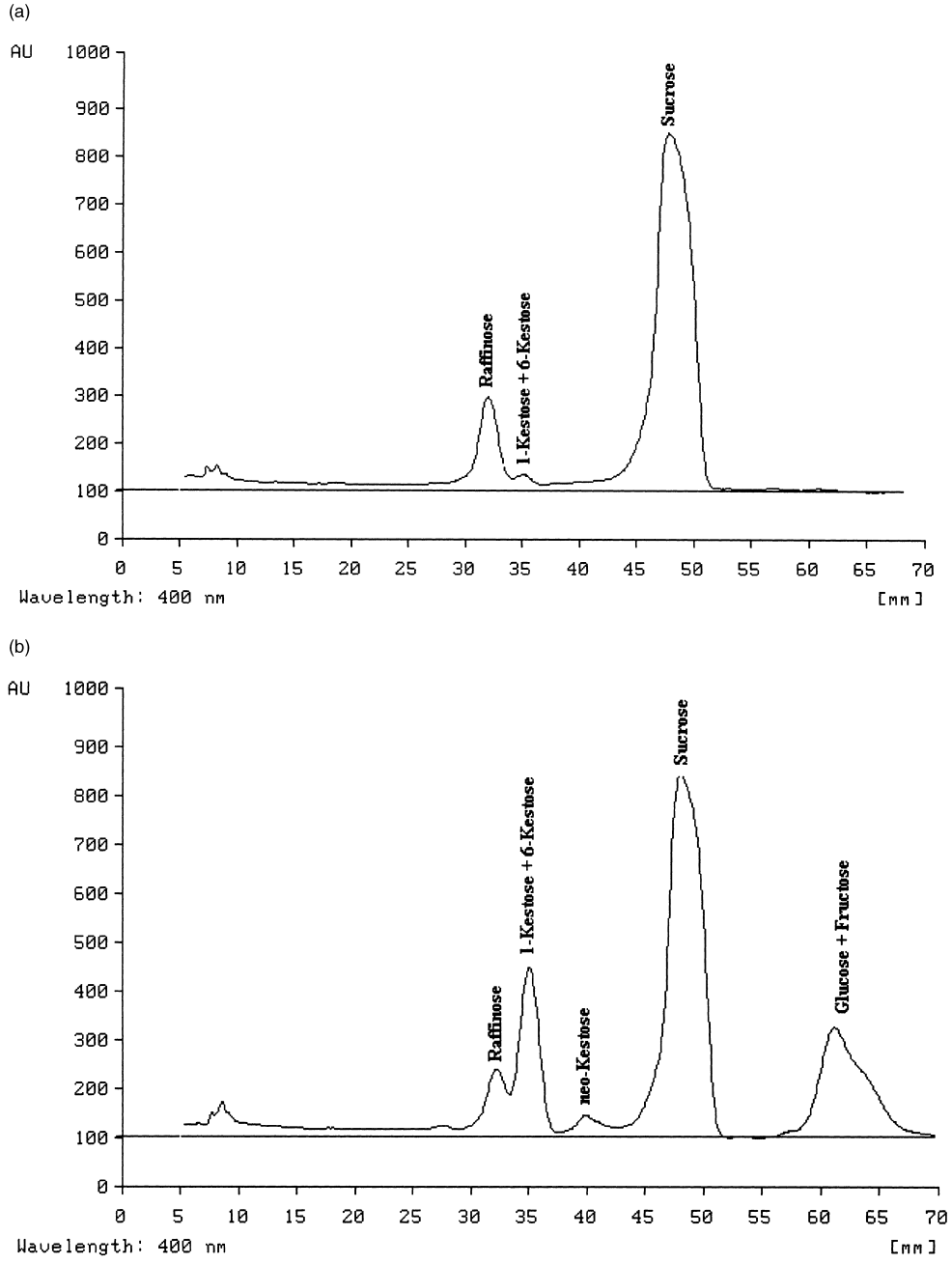


Fig. 9. Densitograms of the molasses obtained from (A) traditional thick juice and from (B) raw juice respectively.

4. Conclusions

The present application shows that modern instrumental TLC allows not only for a rapid screening of several molasses or other samples simultaneously, with no clean-up, but giving a quantitative determination of different oligosaccharides at low nanogram levels.

The quantitative determination of raffinose and kestoses can not only make it possible to obtain a more complete characterization of molasses but also to check particular situations occurring in the sugar factories and expecially in respect of crystallization.

The quantitative determination of fructo-oligosaccharides in natural or in commercial products can give us the opportunity of collecting information on the composition of special raw materials. As an example, transgenic beet which are rich kestoses can be mentioned, as well as commercial mixtures utilized in dietary applications as products showing pre-biotic characteristics.

Table 4
Composition of molasses samples originating from cooling crystallization of traditional thick juice and microfiltered raw juice

Oligosaccharides[a]	Molasses from factory thick juice	Molasses from microfiltered raw juice
Raffinose (%NS)	6.00	3.65
1-kestose + 6-kestose (%NS)	1.20	9.13
Neo-kestose (%NS)	–	0.76

[a] NS, non sucrose.

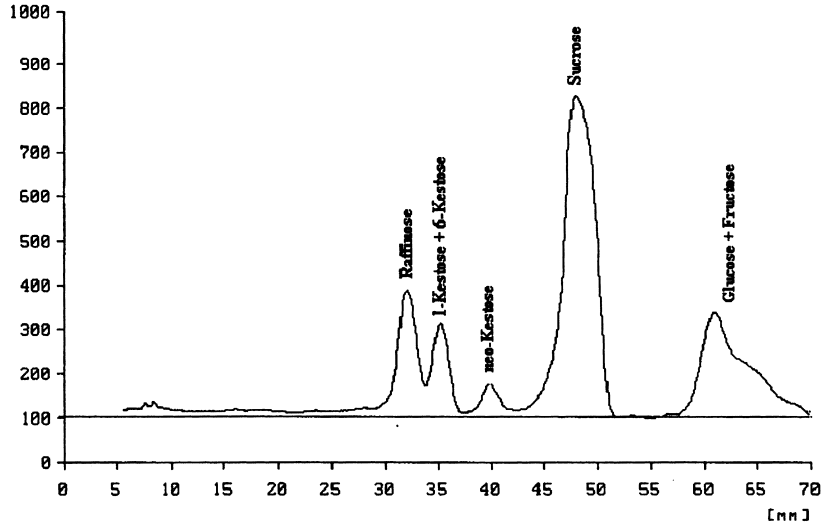


Fig. 10. Densitogram of molasses from chromatographic separation of raw juice.

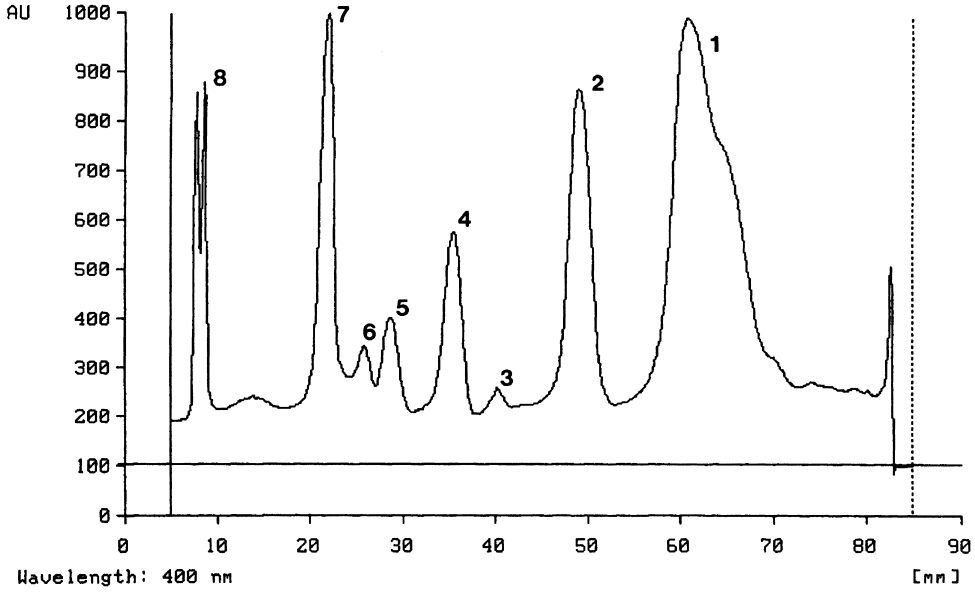


Fig. 11. Densitogram of the mixture of sugars obtained from atichoke leaves. (1) glucose + fructose; (2) sucrose; (3) *neo*-kestose; (4) 1-kestose + 6-kestose; (5) nystose; (6) fructosyl-nystose; (7) inuline-low molecular weight; (8) inulin-high molecular weight.

References

Bacon, J. S. D., & Edelman, J. (1951). The carbohydrates of the Jerusalem artichoke and other compositae. *Biochemical Journal, 48*, 114–126.

Burger, K. D., & Tengler, H. (1986). Automated multiple development — a new technique of thin-layer chromatography. In R. E. Kaiser, *Planar chromatography,* Vol. 1 (pp. 193-205). Heidelberg: Hueting.

Jork, H., Funk, W., Fisher, W., & Wimmer, H. (1990). *Thin layer chromatography*, Vol. 1A. Weinheim: VCH.

Lodi, G., Betti, A., Brandolini, V., Menziani, E., & Tosi, B. (1994). Automated multiple development HPTLC analysis of sugars on hydrophilic layers: I. amino layers. *Journal Planar Chromatography, 7*, 29–33.

Lodi, G., Bighi, C., Brandolini, V., Menziani, E., & Tosi, B. (1997). Automated multiple development HPTLC analysis of sugars on hydrophilic layers: II. Diol layers. *Journal Planar Chromatography, 10*, 31–37.

Mantovani, G., Vaccari, G., Dosi, E., & Lodi, G. (1998). Application of planar chromatography to sample analysis in the sugar industry. *Carbohydrate Polimers, 37*, 263–272.

Quarne, F., & Guibert, A. (1996). Fructo-oligosaccharides: enzymatic synthesis from sucrose. *Zuckerindustrie, 120*(9), 793–798.

Sévenier, R., Hall, R. D., van der Meer, I. M., Hakkert, H. J. C., van Tunen, A. J., & Koops, A. J. (1998). High level fructan accumulation in a transgenic sugar beet. *Nature Biotechnology, 16*, 843–846.

Sungsoo Cho, S., Prosky, L., & Dreher, M. (1999). *Complex carbohydrate in foods*. New York: Marcel Dekker.

Vaccari, G., Mantovani, G., Sgualdino, G., Aquilano, D., & Rubbo, M. (1986). The raffinose effect on sucrose morphology and kinetics. *Sugar Technology Review, 13*, 133–178.

Vaccari, G., Mantovani, G., Sgualdino, G., Tamburini, E., & Aquilano, D. (1999). Fructo-oligosaccharides and sucrose crystal growth morphology. I. experimental growth habit. *Zuckerindustrie, 124*, 34–39.

Vaccari, G., Sgualdino, G., Tamburini, E., Lodi, G., Aquilano, D., & Mantovani, G. (1999). Fructo-oligosaccharides and sucrose crystal growth morphology. II. Verification of nonsucrose absorption through chromatographic analysis and x-ray diffractometry. *Zuckerindustrie, 124*, 536–541.

Vaccari, G., Mantovani, G., Sgualdino, G., Tamburini, E., & Dosi, E. (1999a). Cooling crystallization of raw juice. *Zuckerindustrie, 124*, 947–954.

Vaccari, G., Tamburini, E., Sgualdino, G., Kearney, M., Kochergin, V., & Costesso, D. (2000). Cooling crystallization applied to the "extract" of a chromatographic separation process (SMB) of beet raw juice. In *Proceedings of the CHISA 2000 congress* (CD-rom). Prague: Czech Society of Chemical Engineering.