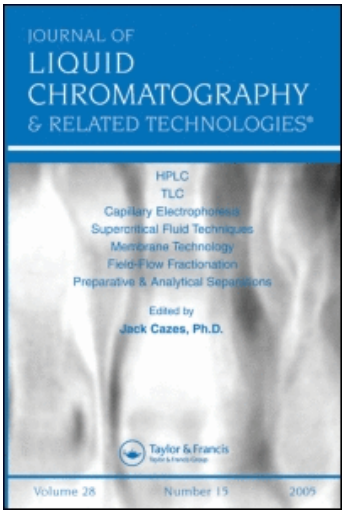

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Simultaneous Identification of Sugars by HPLC Using Evaporative Light Scattering Detection (ELSD) and Refractive Index Detection (RI). Application to Plant Tissues

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SIMULTANEOUS IDENTIFICATION OF SUGARS BY HPLC USING EVAPORATIVE LIGHT SCATTERING DETECTION (ELSD) AND REFRACTIVE INDEX DETECTION (RI). APPLICATION TO PLANT TISSUES

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ABSTRACT

Two detection systems for sugar HPLC identification, were compared, namely IR and ELSD. Peak separation was carried out on an adapted BECKMAN HPLC chromatographic line with the two detectors in series. This assembly line allowed the superimposition of two detection features on an single chromatogram. The same sugar mixture injection should be identified with both systems in the same conditions. Three separation devices were used.

It was demonstrated that the ELSD technique was well adapted for sugar determination. Compared with the RI detection, ESD showed a better sensitivity, more stability of the chromatographic base line and no incidence of the temperature.

However, the main advantage of the ESLD system was the assessment of the gradient elution use, since this is excluded in RI detection.

INTRODUCTION

Sugars extracted from biological tissues can be identified using a number of analytical techniques. Among them gas and

liquid chromatography are well suited to qualitative and quantitative analyses. HPLC is used more commonly.

According to the kinds of sugars in the medium, several types of columns were employed. Until recently the chromatographic detection systems were mainly fluorimetry, UV spectrometry and refractometry, the latter being the most popular.

In spite of its acknowledged qualities, refractometry has some limitations :

- temperature detection needs to be controlled and regulated
- low sensitivity is induced by a high signal/background ratio
- use of gradient elution is excluded.

Recently a new detection system was proposed in liquid chromatography - the Evaporative Light Scattering Detection (ELSD). A number of papers have described this detector and the related chromatographic applications to the analysis of non-volatile compounds [1], [2], [3], [4], [5], [6], [7], [8]. This detection system favorably replaces the refractometry detection for compounds like sugars [9], [10], [11], [12], [13], [14].

The aim of this paper is to compare identification of soluble sugars with a Differential Refractometer (RI) and an Evaporative Light Scattering Detector (ELSD), connected in series, on the same column effluent.

MATERIALS AND METHODS

Chromatographic Equipment

The chromatographic line consisted of the following modules :

- programmable solvent module 126 (System Gold - Beckman Solvent Pumps)
- injection valve, Altex Reference 210A

- analog Interface Module 406 (System Gold - Beckman)
- IBM PC Personal System II (System Gold - Beckman)
- printer EPSON LX 800
- column compartment heater Croco-cil™ Beckman

Detectors

- Differential Refractometer KNAUER
- Evaporative Scattering Detector SEDEX 45 [12]

The liquid phase is transformed into an aerosol in which the volatile constituents are easily evaporated. The homogeneous aerosol produced has non volatile components for quantitative identification.

Column choice

Three types of columns were tested. A guard column was systematically used to protect the column in the separation devices as follows :

n°1 : - Guard column : RP18 5 µm, Lichrosorb MERCK 60 mm
- Column R-NH_2, type Lichrospher MERCK 100 NH_2-5 µm
HPLC Cartridge LichroCART ref 50834 250-4 mm

n°2 : - Guard column : Polyspher Guard Column CH-Pb 20-3 mm
MERCK ref 51275
- Column : Polyspher CH-Pb 300-7.8mm
MERCK ref 51278

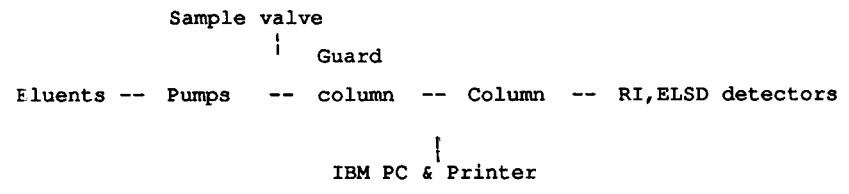
n°3 : - Guard column : Polyspher Guard Column CH-Pb 20- 3 mm
MERCK ref 51279
- Column : AMINEX Carbohydrate HPX - 87C(Ca) 300- 7.8 mm
BIORAD ref 1250095

Reference Sugars

The choice of reference sugars was directed by the search for free sugar contents in coniferous tree foliar tissues. Sugars identified were : rhamnose, xylose, fructose, glucose, sucrose, maltose, melibiose, melezitose, raffinose and the related compounds inositol and pinitol.

Chromatographic line scheme

The chromatographic line scheme is as follows :



The experimental chromatographic line used in this analysis was with RI and ELSD in series. This scheme allowed the superimposition of two detection modes on a single chromatogram corresponding to the injection of the same sugar mixture in the same conditions.

RESULTS AND DISCUSSION

The analysis of chromatographic data is based on the system described above. The chromatographic characteristics discussed are as follows :

- base line drift (stability)
- sensitivity

- isocratic mixed phase chromatography
  with pump assistance
  manual mixing before chromatography
- gradient chromatography.

Base line drift stability

The RI system showed a base line drift affected by temperature variation. Thermoregulation is needed if high sensitivity of the HPLC system is required. Experiments could be performed only when stabilization of RI system was reached. Stability depended on the mobile phase and of the columns' characteristics (FIGURES 1, 3, 5).

On the other hand, ELSD identification exhibited a stable base line, independent of temperature changes. The system can be used without stabilization time (FIGURES 1 and 2).

Sensitivity

ELSD sensitivity was better than refractometric sensitivity. In the ELSD system 10 nmoles of different sugars were identified with accuracy whereas refractometry did not allow this (FIGURE 2). Refractometric sensitivity depended on the mobile phase and of the column characteristics. Peak identification on the polyspher CH-Pb using a water mobile phase showed a better refractometric sensitivity and a better base line stability (FIGURES 1 and 3).

Isocratic chromatography in a mixed phase with assistance of several pumps

When high sensitivity of refractometric detection is required, the automatic mixing of solvent phases can lead to

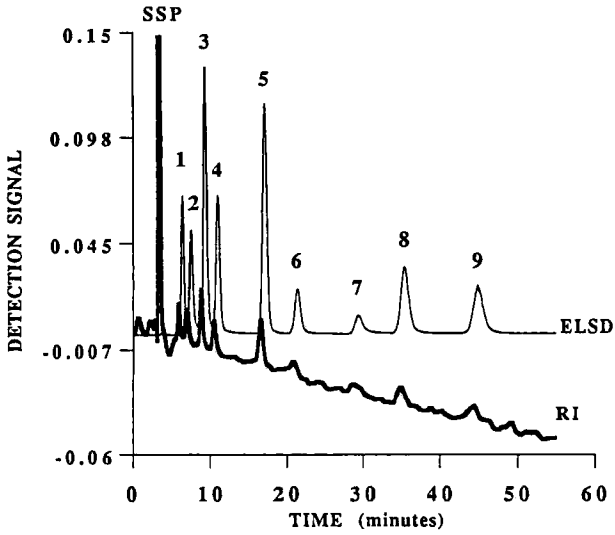


FIGURE 1. Sugar separation on R-NH2 column

References :		Retention time ELSD (minutes)	Quantities injected (µ moles)
SSP	Sample Solvent Peak, (water)		
1 C5(CH3)	Rhamnose	6.5	0.26
2 C5	Xylose	7.5	0.31
3 C6	Fructose	9.3	0.33
4 C6	Glucose	11.0	0.26
5 C12	Sucrose	17.1	0.23
6 C12	Maltose	21.3	0.12
7 C12	Melibiose	29.4	0.12
8 C18	Melezitose	35.4	0.11
9 C18	Raffinose	44.9	0.10

Chromatographic characteristics :

- Guard Column : RP18 - 5 µm Lichrosorb MERCK 60 mm
- Column : Lichrospher 100 NH2 - 5 µm MERCK HPLC Cartridge Lichrocart 250-4 mm, ref. 508342504
- Sample Loop : 20 µl
- Eluent : Acetonitrile / Water (80 / 20 V), automatic pumps mixing
- Flow rate : 1 ml / minute
- Column Temp. : 25°C
- Detection : - RI, Differential Refractometer KNAUER, (Range 4)
  - ELSD, Evaporative Light Scattering Detector SEDEX 45, (Gain 5 - Temperature 40°C - Pressure 2.5 Bars)

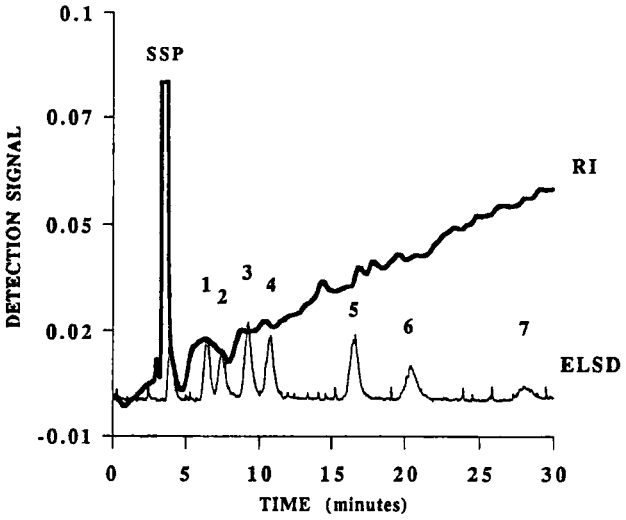


FIGURE 2. Sugar separation on R-NH2 column

References :		Retention time ELSD (minutes)	Quantities injected (nmoles)
SSP	Sample Solvent Peak, (water)		
1 C5(CH3)	Rhamnose	6.4	12.8
2 C5	Xylose	7.5	14.0
3 C6	Fructose	9.2	11.8
4 C6	Glucose	10.8	11.9
5 C12	Sucrose	16.6	6.54
6 C12	Maltose	20.4	5.88
7 C12	Melibiose	29.5	5.91

Chromatographic characteristics :

- Guard Column	:	RP18 - 5 µm Lichrosorb MERCK 60 mm
- Column	:	Lichrospher 100 NH_2 - 5 µm MERCK HPLC Cartridge Lichrocart 250-4 mm, ref. 508342504
- Sample Loop	:	20 µl
- Eluent	:	Acetonitrile / Water (80 / 20 V), automatic pumps mixing
- Flow rate	:	1 ml / minute
- Column Temp.	:	25°C
- Detection	:	- RI, Differential Refractometer KNAUER, (Range 4) - ELSD, Evaporative Light Scattering Detector SEDEX 45, (Gain 7 - Temperature 40°C - Pressure 2.5 Bars)

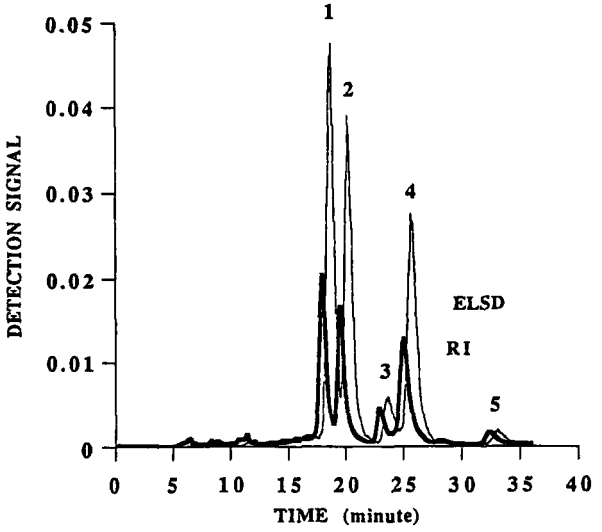


FIGURE 3. Sugars in pine buds - Separation on CH - Pb Column

Sugars and related compounds *		Retention time ELSD (minutes)	Concentration (µmoles)
1 C6	Glucose	18.6	0.19
2 C6(CH3)	Pinitol *	20.2	0.14
3 ---	Unknown	23.6	-----
4 C6	Fructose	25.8	0.20
5 C6	Inositol *	33.6	0.02

Chromatographic characteristics :

- Guard Column : Polyspher Guard Column CH - Pb 20 -3 mm, MERCK ref. 51279
- Column : Polyspher CH - Pb 300 -7.8 mm, MERCK ref. 51278
- Sample Loop : 20 µl
- Eluent : Water
- Flow rate : 0.4 ml / minute
- Column Temp. : 80°C
- Detection : - RI, Differential Refractometer KNAUER, (Range 16)
  - ELSD, Evaporative Light Scattering Detector SEDEX 45,
  (Gain 6 - Temperature 45°C - Pressure 3.0 Bars)

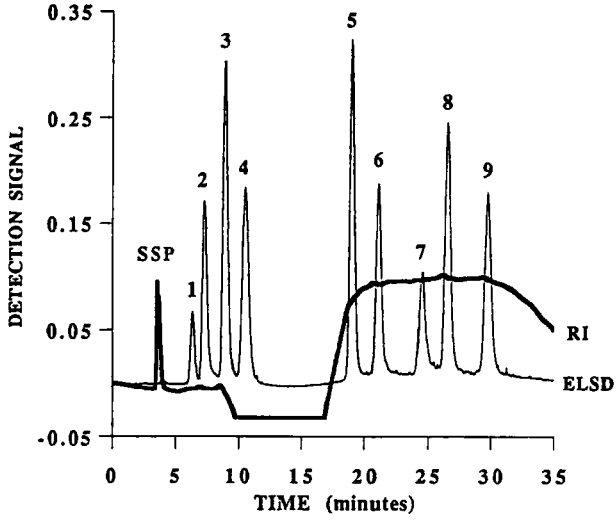


FIGURE 4. Sugar separation on R-NH2 column using a gradient

References :		Retention time ELSD (minutes)	Quantities injected (µmoles)
SSP	Sample Solvent Peak, (water)		
1 C5(CH_3)	Rhamnose	6.3	0.26
2 C5	Xylose	7.3	0.31
3 C6	Fructose	8.9	0.33
4 C6	Glucose	10.5	0.26
5 C12	Sucrose	18.9	0.23
6 C12	Maltose	21.1	0.12
7 C12	Melibiose	24.6	0.12
8 C18	Melezitose	26.6	0.11
9 C18	Raffinose	29.8	0.10

Chromatographic characteristics :

- Guard Column	:	RP18 - 5 µm Lichrosorb MERCK 60 mm
- Column	:	Lichrospher 100 NH_2 - 5 µm MERCK HPLC Cartridge Lichrocart 250-4 mm, ref. 508342504
- Sample Loop	:	20 µl
- Eluent	:	Acetonitrile / Water, automatic pumps mixing
- Gradient	:	0 - 4 minutes 80 / 20 V constant 4 - 8 minutes 80 / 20 V to 88 / 12 V linear 8 - 17 minutes 88 / 12 V to 75 / 25 V linear 17 - 21 minutes 75 / 25 V constant 21 minutes 75 / 25 V to 80 / 20 V promptly 21 - 35 minutes 80 / 20 V constant
- Flow rate	:	1 ml / minute
- Column Temp.	:	25°C
- Detection	:	- RI, Differential Refractometer KNAUER, (Range 4) - ELSD, Evaporative Light Scattering Detector SEDEX 45, (Gain 5 - Temperature 40°C - Pressure 2.5 Bars)

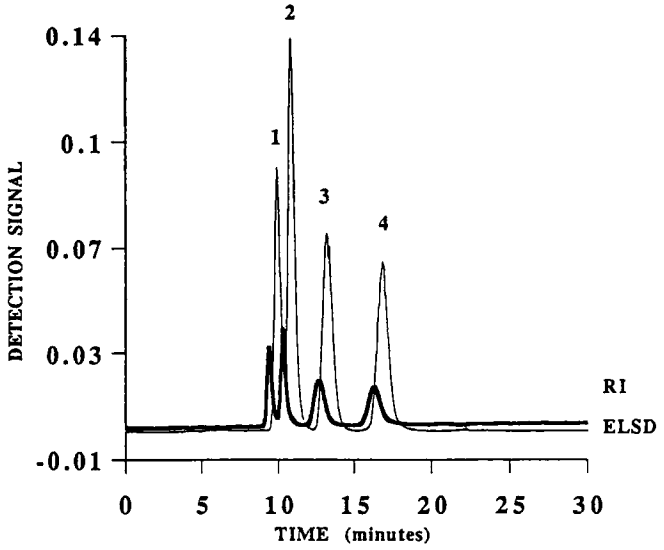


FIGURE 5. Sugar separation on CH - Ca column

References :		Retention time ELSD (minutes)	Quantities injected (µmoles)
1 C18	Raffinose	9.9	0.03
2 C12	Saccharose	10.8	0.06
3 C6	Glucose	13.2	0.11
4 C6	Fructose	16.8	0.11

Chromatographic characteristics :

- Guard Column	:	Polyspher Guard Column CH - Pb 20 -3 mm, MERCK ref. 51279
- Column	:	Aminex Carbohydrate HPX - 87C (Ca) 300 -7.8 mm, BIORAD ref. 1250095
- Sample Loop	:	20 µl
- Eluent	:	Water
- Flow rate	:	0.5 ml / minute
- Column Temp.	:	80°C
- Detection	:	- RI, Differential Refractometer KNAUER, (Range 8) - ELSD, Evaporative Light Scattering Detector SEDEX 45, (Gain 7 - Temperature 48°C - Pressure 3.0 Bars)

variations of the base line, induced by a non homogeneous mixed phase. To avoid this situation, manual mixing could be carried out before chromatography (FIGURE 3). No base line variations were observed when the ELSD system was used, since the mixed phase was evaporated before the sugar detection.

Gradient chromatography

To improve peak selection and to reduce elution time, gradient elution is commonly used in modern chromatography. ELSD allowed the use of gradients, while this parameter is inapropriate for the RI detector (FIGURE 5).

We selected a complicated gradient to prove the independance of the ELSD method from the gradient of the solvent (FIGURE 4). A different RI detection signal appeared for the same mobile phase used at the begining and at the end of the chromatography and showed base line drift.

CONCLUSION

The Evaporative Light Scattering Detector appears as a promising detector for HPLC of non-volatile compounds, like sugars. Compared to the RI detection, the ELSD detection shows at least three advantages :

- it brings more stability on the chromatographic base line
- has a better sensitivity in the sugar detection
- and allows use of gradient elution.

As it is easy to set up on a chromatographic HPLC line, the ELSD detector is a complementary and useful tool and may replace the RI detector for sugar identification in vegetal tissues

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