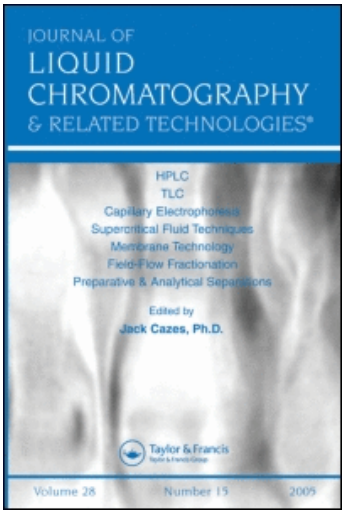

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Determination of Sugars in Tobacco Leaf by HPLC with Evaporative Light Scattering Detection

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Abstract: A reversed phase liquid chromatography-evaporative light scattering detection, combined with solid phase extraction method was developed for the separation and determination of sugars in tobacco leaf. Separation was performed with a Hypersil NH_2 analytical column and the mobile phase 75%:25% acetonitrile-water. In the range of 0.1–4 mg/mL, the method showed good linearity, with the correlation coefficients exceeding 0.99. The limits of detection of xylose, fructose, glucose, sucrose, maltose, and raffinose were 2, 2, 3, 2, 4, and 4 μg/mL, respectively. The relative standard deviations of peak areas for these sugars were in the range of 2–3%. Recoveries of fructose, glucose, and sucrose were between 96 and 117%. Finally, determination of sugars in tobacco leaf, before and after bio-catalysis, was performed with this method.

Keywords: Solid-phase extraction, Liquid chromatography, Evaporative light scattering, Tobacco leaf, Sugars

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INTRODUCTION

Sugars are significant constituents of tobacco leaf. They contribute to smoke flavor and have a positive influence on tobacco quality, and they appear in higher concentrations in desirable leaves. Sugars and amino acids can form Maillard products that can be broken down into flavor related compounds in the tobacco leaf mellowing process. They can also be combined with alcohols, phenols, polyphenols, and sterols to form glycosides which contribute to smoke flavor. Among the soluble sugars, fructose and glucose are the most important, and they are known by a common name, i.e., reducing sugar. In general, the leaf with a greater concentration of reducing sugar is regarded as the one with a better smoking quality.[1–6] By the application of a biocatalyst, oligosaccharides and polysaccharides can be transformed into the reducing sugar to improve the quality of tobacco leaf. Therefore, reliable determination of these sugars in tobacco leaf is essential for the classification of tobacco crude materials.[15]

Some authors determined sugars with a colorimetric method,[7] thin-layer chromatography,[8] and gas chromatography.[9,10] Many authors used high-performance liquid chromatography (HPLC) for rapid characterization of sugars.[11–22] Sugars can be detected directly within the range of 185–192 nm using a UV detector. But, the sensitivity has been poor, due to the absence of a strong chromophore. After appropriate derivatization, fluorescence detection was used.[11–13] The most common method for the quantification of sugars by HPLC analysis has been refractive index (RI) detection.[14–16] But, RI detection cannot be used in combination with gradient elution, and it also lacks high sensitivity. Electrochemical detection, in particular, the common PAD, was also used in analysis of sugars.[17] The evaporative light-scattering detector is a universal detector and it has high sensitivity for non-ultraviolet absorbing compounds, and it is sensitive for non-volatile solutes in a volatile liquid stream.[18,19]

Here, we describe an SPE-HPLC-ELSD method for analysis of sugars in tobacco leaf. The content of sugars, before and after bio-catalysis of tobacco leaf, was analyzed to test the usefulness of the method.

EXPERIMENTAL

Instrument

All chromatographic experiments were conducted using a Shimadzu LC system (Shimadzu Scientific Instruments, Japan) incorporating an LC-10Atvp pumping system, an SIL-10Advp auto-injector (with a 20 μL loop), an SCL-10Avp system controller, a DGU-12A Degasser, and an ELSD 2000 evaporative light-scattering detector (Alltech, USA). The analytical column (4.6 mm × 250 mm) was packed with 5 μm Hypersil NH2 (Elite,

Dalian, China). The solid phase extraction system used was from J&W Scientific, USA and rotary evaporator was from Shanghai, China.

Chemicals and Tobacco Leaf Samples

Sugars were analytical grade (Shanghai, China), pentaerythritol was chemical grade (Dalian, China), methanol and acetonitrile were HPLC grade (Tedia, USA). Water was deionised using a Milli-Q water purifier.

Sucrase was prepared in our lab. Maltase, cellobiase, amylase, complex cellulose, and complex pectinase were obtained from Novozymes in China.

The tobacco leaf samples used were obtained from Chuxiong Cigarette Company (Yunnan, China).

Preparation of Standards and Samples

The stock solutions of all sugars (4 mg/mL) and pentaerythritol (5 mg/mL) were prepared by dissolving 200 mg of sugars and 250 mg of pentaerythritol standard in Milli-Q water.

Pretreatment of samples: The dry leaf was ground to pass a 40 mesh screen prior to extraction. A 10 mL volume of the extraction solution containing 0.5 mL acetic acid, 2 mL methanol, 0.5 mL of pentaerythritol, and 7.0 mL Milli-Q water was poured over 500 mg of sample, and extraction was carried out in an ultrasonic bath for 5 min at room temperature. Sugars were analyzed in 1.5 mL aliquots of extract.

Acetonitrile, 8.5 mL, was added into the 1.5 mL extract solution and the sample was passed through an NH_2 cartridge, preceded by activation of the sorbent with 5 mL of methanol and 5 mL of acetonitrile-water (4:1, v:v). Sugars were eluted from the cartridge with 3 mL of acetonitrilbe-water (1:1, v:v). After evaporation, the residue was redissolved in 1 mL Milli-Q water and was then injected into the LC system for analysis.

HPLC-ELSD Conditions and Quantification

The influence of the tube temperature and gas flow rate setting on the detector signal was investigated. First, the tube temperature was set to 81.3°C and the gas flow rate was set to 2.1 L/min), according to the mobile phase. The flow rate was fixed and the temperature was changed until the baseline was steady and the noise was within a very narrow range. We found that 81.3°C was the best temperature. Then the temperature was fixed and the flow rate was changed. The largest signal response was achieved when the flow rate was 1.9 L/min.

Chromatographic separation was carried out with an isocratic elution mobile phase of acetonitrile-water (75:25, v:v). The flow rate was 1.0 mL/min

and the volume of the sample injected was 10 μL. Column temperature was set at room temperature. Peaks were identified by both retention time and the standard addition method. Peaks were quantified by an internal standard method.

RESULTS AND DISCUSSION

Selection of Extraction Solvent and SPE Condition

Water and alcohols have often been used in the extraction of sugars, but these methods are time consuming.[20] We found that addition of some weaker acids, such as acetic acid, into the extraction solution can quicken the extraction of sugars. The extraction was completed after only 5 min of sonication at room temperature.

Many kinds of organic compounds in tobacco leaf can easily be extracted with sugars and are very likely to interfere with the subsequent chromatographic analysis. Hence, a selective isolation procedure for sugars is necessary.

Solid phase extraction (SPE) is widely used in pretreatment of complex samples. In this work, an NH_2 cartridge was used to isolate sugars from tobacco. By testing the proportion (0%, 25%, 50%, and 75%) of acetonitrile in the sample, we found that the peak area of 75% was four times the others. With an eluent of 50:50 acetonitrile-water, not only all sugars were eluted from the cartridge, but also less interference was found.

Selection of LC Conditions

For the determination of the sugars by the LC method, silica gel derivatised with amino groups is the most widely used stationary phase. The selection can be adjusted by changing polarity and composition of the mobile phase to obtain the desired separation. By testing the mobile phases in the proportion 70:30, 75:25, 80:20, 85:15, and 90:10, we found that, with a 75:25 of acetonitrile-water, the sugars from the tobacco leaf were rapidly eluted and the peaks were separated well. Typical chromatogram of sugar standards and a tobacco leaf sample are shown in Fig. 1.

Analytical Characteristics of the Method

Linearity

A linear relationship between the concentration of sugars and relative peak area (sugars to internal standard) was found. For all these sugars, the R^2 values exceeded 0.99 at eight levels (Table 1).

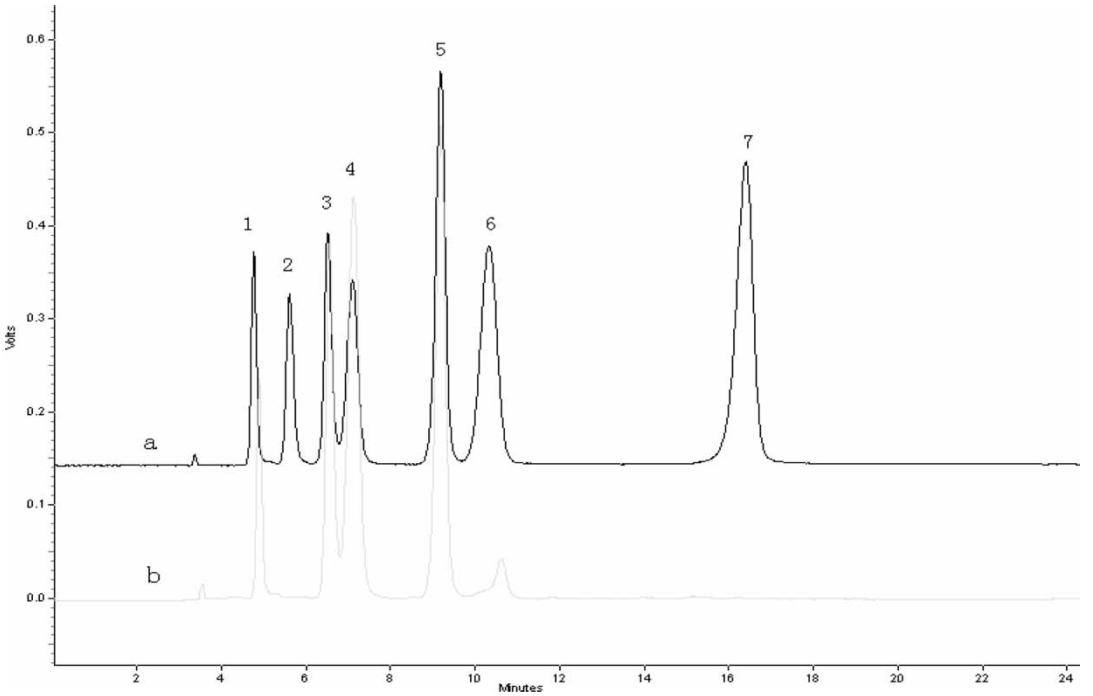

Figure 1. Chromatograms of mixed sugar standards (a) and tobacco leaf sample (b). Mobile phase-acetonitrile-water (75:25, v:v), Column temp. is at room temperature. Peaks: 1, pentaerythritol (internal standard); 2, xylose; 3, fructose; 4, glucose; 5, sucrose, 6, maltose; 7, raffinose.

Table 1. Linearity of sugar determination by SPE-HPLC-ELSD method

Compound	R^{2a}	Calibration curve[b]
Xylose	0.996	$y = 0.001x - 0.126$
Fructose	0.998	$y = 0.0015x - 0.1425$
Glucose	0.997	$y = 0.0017x - 0.1944$
Sucrose	0.999	$y = 0.0029x - 0.1274$
Maltose	0.997	$y = 0.003x - 0.2974$
Raffinose	0.999	$y = 0.0038x + 0.0113$

The concentration of the standards was in the range of 0.1–4 mg/mL.

[a]Linear coefficients of working curve.

[b]The linear correlations were obtained by eluting the eight standard aqueous sugar solutions with different concentrations. y is the relative peak area of sugars to internal standard, x is the concentration of sugar standards.

Sensitivity and Recovery

To check the sensitivity of this method, the limit of detection (LOD) was studied. It is clear that ELSD is more sensitive than RID.[16]

Recoveries were obtained by adding sugar standards to sample before pretreatment. The results are shown in Table 2.

Repeatability

Six consecutive injections were carried out, both in the same day and between different days, to evaluate the repeatability. The results are listed in Table 3.

Table 2. Detection limits and recovery of sugars by SPE-HPLC-ELSD method

Compound	LOD[a] (μg/mL)	Recovery[b]
Xylose	2	
Fructose	2	109 ± 6
Glucose	3	117 ± 5
Sucrose	2	96 ± 3
Maltose	4	
Raffinose	4	

[a]Calculated based on three signal to noise ratio.

[b]Fructose 3.00 mg, glucose 3.75 mg, sucrose 3.75 mg were added.

Table 3. Repeatability of peak area for standard sugars

Compound	Intra-day mean[a] (10^5)	RSD[b] (%)	Inter-day mean[a] (10^5)	RSD[b] (%)
Xylose	131	2.54	136	3.28
Fructose	161	3.16	167	3.75
Glucose	101	2.89	106	3.62
Sucrose	127	2.53	132	3.82
Maltose	105	2.80	109	4.05
Raffinose	89	3.33	92	4.17

[a]Mean value of peak area.
[b]Relative standard deviation of peak area.

Determination with Tobacco Leaf Samples

It has been known that some chemical components of tobacco leaf, such as starch and protein, have a negative influence on tobacco and smoke quality. Through bio-catalysis, these components can be decomposed into reducing sugars and amino acids that contribute positively to tobacco quality. A certain proportion of enzymes were added before the mellowing process to facilitate tobacco fermentation. To test the usefulness of the SPE-HPLC-ELSD method, analysis of sugars in tobacco leaf, before and after bio-catalysis, was carried out.

The results are listed in Table 4. The content of reducing sugars increased while the level of sucrose decreased after bio-catalysis. The effect of multi-enzymes was better than that of single enzymes.

The smoking quality of tobacco (treated and untreated by biocatalyst) was also evaluated by the organoleptic method. All the members of the expert

Table 4. Determination of sugars in tobacco leaf before and after bio-catalytic reaction

Enzyme	Fructose (%)	Glucose (%)	Sucrose (%)
None	9	14	6
Sucrase	10	16	4
Maltase	9	13	6
Cellobiase	10	16	4
Amylase	10	16	5
Complex cellulase	10	17	4
Complex pectinase	10	16	5
Multi-enzyme1	11	17	4
Multi-enzyme2	11	18	4
Multi-enzyme3	12	18	4

panel deemed that the taste and the quality of the leaf had significant improvement after bio-catalysis.

CONCLUSIONS

An SPE-HPLC-ELSD method for the determination of sugars in tobacco leaf has been developed. The method shows good sensitivity and repeatability, and it is suitable for routine analysis of sugars in tobacco leaf. The content of reducing sugars was determined before and after bio-catalysis. All reducing sugars had increased by 15–33% after bio-catalysis.

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REFERENCES

1. Mendell, S.; Bourlas, E.C.; DeBardeleben, M.Z. Factors influencing tobacco leaf quality: an investigation of the literature. Beitrage zur Tabakforschung Int. **1984**, *12* (3), 153–167.
2. Tso, T.C.; Gori, C.B. Leaf quality and usability: theoretical model. Beitrage zur Tabakforschung Int. **1975**, *8* (4), 167–173.
3. Cambell, J.S. Trends in tobacco leaf usability. Beitrage zur Tabakforschung Int. **1995**, *16* (4), 185–195.
4. Hasebe, H.; Subara, S. The quality estimation of different tobacco type examined by headspace vapor analysis. Beitrage zur Tabakforschung Int. **1999**, *18* (5), 213–222.
5. Smeeton, B.W. Genetic control of tobacco quality. Rec. Adv. Tobac. Sci. **1987**, *13*, 3–26.
6. Wei, M.L.; Sung, J.M. Carbohydrate metabolism enzymes in developing grains of rice cultured in solution with calcium supplement. Crop Sci. **1993**, *33*, 174–177.
7. Rodríguez-Sevilla, M.D.; Villanueva-Suárez, M.J.; Redondo-Cuenca, A. Effects of processing conditions on soluble sugars content of carrot, beetroot and turnip. Food Chem. **1999**, *66*, 81.
8. Han, N.S.; Robyt, J.F. Separation and detection of sugars and alditols on thin layer chromatograms. Carbohyd. Res. **1998**, *313*, 135.
9. Adams, M.A.; Chen, Z.L.; Landman, P.; Colmer, T.D. Simultaneous determination by capillary gas chromatography of organic acids, sugars, and sugar alcohols in plant tissue extracts as their trimethylsilyl derivatives. Anal. Biochem. **1999**, *266*, 77.

10. Silva, F.O.; Ferraz, V. Microwave-assisted preparation of sugars and organic acids for simultaneous determination in citric fruits by gas chromatography. Food Chem. **2004**, *88*, 609.
11. Diaz, J.; Liberia, J.; Comellas, L.; Broto-Puig, F. Amino acid and amino sugar determination by derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate followed by high-performance liquid chromatography and fluorescence detection. J. Chromatogr. A **1996**, *719*, 171.
12. Huang, Z.; Prickett, T.; Potts, M.; Helm, R. The use of the 2-aminobenzoic acid tag for oligosaccharide gel electrophoresis. Carbohyd. Res. **2000**, *328*, 77.
13. Tran, N.; Daali, Y.; Cherkaoui, S.; Taverna, M.; Neeser, J.; Veuthey, J. Routine *o*-glycan characterization in nutritional supplements—a comparison of analytical methods for the monitoring of the bovine kappa-casein macropeptide glycosylation. J. Chromatogr. A **2001**, *929*, 151.
14. Lenherr, A.; Mabry, T.J.; Gretz, M.R. Differentiation between glucose, mannose, allose and galactose in plant glycosides by high-performance liquid chromatographic analysis. J. Chromatogr. **1987**, *388*, 455.
15. Svob Troje, Z.; Frobe, Z.; Perovic, D. Analysis of selected alkaloids and sugars in tobacco extract. J. Chromatogr. A **1997**, *775*, 101.
16. Chavez-Servin, J.L.; Castellote, A.I.; Lopez-Sabater, M.C. Analysis of mono- and di-saccharides in milk-based formulae by high-performance liquid chromatography with refractive index detection. J. Chromatogr. A **2004**, *1043*, 211.
17. Schiller, M.; von der Heydt, H.; Schmidt, P.C. Quantification of sugars and organic acids in hygroscopic pharmaceutical herbal dry extracts. J. Chromatogr. A **2002**, *968*, 101.
18. Lucena, R.; Cárdenas, S.; Gallego, M.; Valcárcel, M. Continuous flow autoanalyzer for the sequential determination of total sugars, colorant and caffeine contents in soft drinks. Anal. Chim. Acta **2005**, *530* (2), 283.
19. Kumar Chitneni, S.; Govaerts, C.; Adams, E.; Van Schepdael, A.; Hoogmartens, J. Identification of impurities in erythromycin by liquid chromatography—mass spectrometric detection. J. Chromatogr. A **2004**, *1056*, 111.
20. Ramusino, M.C.; Dattlio, B.S.; Lucibello, A. Beitrage zur Tabakforschung Int. **1994**, *16* (2), 77.
21. Molnar-Perl, I. Role of chromatography in the analysis of sugars, carboxylic acids and amino acids in food. J. Chromatogr. A **2000**, *891*, 1.
22. Wang, Q.; Fang, Y. Analysis of sugars in traditional Chinese drugs. J. Chromatogr. B **2004**, *812*, 309.

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