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Optimization of carbohydrate silylation for gas chromatography

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Abstract

We developed and optimized a new carbohydrate mono- and disaccharides silylation reaction, replacing pyridine and requiring lower reaction temperature and less time. Our method consists of three basic steps. The first one is oxime formation, the second one silylate derivative and the last one gas chromatography separation and quantification with an internal standard. We evaluated several solvents, including acetonitrile, hydroxylamine and aniline. We found aniline to be the best reaction media for oxime formation with hydroxylamine hydrochloride. Among silylation agents we found *N*,*O*-bis(trimethyl)trifluoroacetamide (BSTFA) was the most efficient. Together these reagents favored both a short analysis time and fewer by-products. We evaluated the method with model solutions containing: arabinose and co-eluting xylose, fructose, glucose, sucrose and salicin (internal standard) and found it suitable for processed food analysis.

Keywords: Derivatization, GC; Food analysis; Carbohydrates

1. Introduction

For sugars identification and quantitation either high-performance liquid chromatography (HPLC) [1,2], enzymatic [3] and gas chromatography (GC) [4–8] procedures have been described. All these methods are time consuming and suffer numerous limitations.

GC analyses of sugars require chemical derivatization to produce a volatile molecule. Many derivatization methods for sugars exist, but the simplest and most rapid for routine analysis is silylation to produce trimethylsilyl (TMS) derivatives.

Bentley and Botlock [9] found that aqueous sugar solution produce less mutarotation when mixed with *N*,*N*-dimethylformamide and frozen with liquid nitrogen prior to derivatization.

Sugars quantification as their TMS derivatives has been extended in order to identify and quantify sugar constituents in the hydrolyzates of various processed foods [10].

Other works [7,8,11] demonstrated TMS oximes (Oxi-TMS) analysis is better than TMS methoximes (Methoxi-TMS) derivates, the evaluation was based upon (i) the stability, (ii) the efficiency, (iii) number of by-products formed and (iv) the constant ratio of *syn*- and *anti*-anomeric forms of oximes.

Customary method for oxime preparation involves treatment of carbonyl compounds with hydroxylamine hydrochloride in a basic aqueous medium with pH adjustment. Solvent and reagent amounts and conditions are critical for successful oxime formation [12,13].

Our present study optimizes sugars analysis testing different solvents, reagents to free hydroxylamine from its hydrochloride and silylating reagents. Reaction conditions were optimized for each case.

2. Experimental

2.1. Reagents

All chemicals were of analytical reagent grade. Aniline, *N*,*N*-dimethylformamide, hydroxylamine, hydroxylamine hydrochloride ($NH_2OH{\cdot}HCl$), pyridine and sodium acetate were from Merck (Mexico).

Hexamethyldisilazane (HMDS), *N*,*O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA), trimethylchlorosilane (TMCS), trifluoroacetic acid (TFA) and acetonitrile (ACN) were obtained from Sigma (St. Louis, MO, USA).

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Arabinose, fructose, glucose, salicin, sucrose and xylose were also obtained from Sigma.

Commercial juice was obtained from a grocery store, and natural juice was obtained in our laboratory from fresh fruit. These two samples were analyzed without further treatment.

2.2. Gas chromatography

The apparatus was a Hewlett-Packard 5890 series II gas chromatograph (Folsom, CA, USA) equipped with split injector and flame ionization detection (FID) system, their temperatures were 250 and 320 °C, respectively. The column was a J&W Scientific DB-5, 30 m × 0.25 mm i.d. with 0.25 μm film thickness (Folsom, CA, USA). Oven initial temperature was 160 °C for 1 min, then increased to 172 °C at 2 °C min^{-1} then it was increased at 10 °C min^{-1} until the temperature reached 210 °C, finally it was increased at 40 °C min^{-1} until the temperature reached 310 °C and was held for 2 min. Carrier gas was hydrogen at a flow-rate of 2.7 ml min^{-1} and split ratio was 1:33.

2.3. Sample preparation

Five grams of fruit (pulp) was cut in small pieces and mixed with 12.5 ml water, heated until boiling and cooled to room temperature; 10 ml of 96% ethanol were added and sample was filtered. Final solution was evaporated to dryness under nitrogen to obtain sample extract.

2.4. Oxime preparation

Following works from Streeter and Strimbu [14] and Adams et al. [15] hydroxylamine was used for oxime preparation, original conditions were modified.

We studied several solvents with varying hydroxylamine hydrochloride concentration from 20 to 50 mg/ml, temperature was varied from 60 to 80 °C and reaction time from 5 to 120 min.

For the basic solvents pyridine, aniline and dimethylformamide, solid hydroxylamine hydrochloride was directly dissolved. For acetonitrile, aqueous hydroxylamine (50%) was added for oxime formation. Acetonitrile/water mixture was evaporated and products dissolved in pure acetonitrile.

Hydroxylamine hydrochloride dissolved in aqueous sodium acetate was also tested as reaction medium. Water was evaporated and products were also redissolved in pure acetonitrile.

Ten milligrams of standard sugar mixture, or sample extract, including salicin as internal standard was mixed with 1 ml of hydroxylamine hydrochloride solution in a reaction vial, closed and heated. Resulting solution is cooled to room temperature.

2.5. Silylation reaction

After oxime formation, TMS oxime derivatives (Oxi-TMS) were prepared. Silylating reagent is added, mixed with model or sample solution, the vial is closed and heated. Silylating agent (HMDS/TFA or BSTFA) and mixing were also varied. Both magnetic stirring and ultrasonic mixing were tested.

2.6. Chromatographic analysis

One microliter of silylate derivative (model or sample solution) was injected to the chromatograph. Analytes identification was carried out by retention time comparison and co-injection with model solutions. Number of peaks and their ratio were obtained for the Oxi-TMS of every sugar. Quantitative analysis of sugar silylates were based on relative area response interpolating in respective calibration curve, using salicin as internal standard. Fructose and glucose gave two peaks each one (α,β-anomers).

3. Results and discussion

3.1. Oxime formation

Optimum hydroxylamine hydrochloride concentration for oxime formation was about 50 mg/ml. Table 1 shows optimum temperatures and reaction times for all the tested solvents. Optimum reaction time was obtained from a reaction kinetics plot.

3.2. Silylation reaction

Both HMDS/TFA and BSTFA were tested for Oxi-TMS formation. HMDS/TFA always produced many by-products, even at low temperatures and short reaction times, whereas BSTFA gave only the expected products. Table 2 lists optimum conditions for BSTFA silylation, dimethylformamide is not included there because no reaction was observed with this solvent.

Fig. 1 shows typical chromatogram using hydroxylamine hydrochloride in pyridine and BSTFA, while Fig. 2 shows results using aniline as solvent, noticeably TMS formation in this solvent occurs at ambient temperature. In contrast,

Table 1
Optimum reaction conditions for different solvents used in oxime formation

Solvent	Reaction temperature (°C)	Reaction time (min)
Pyridine	80	30
Aniline	60	10
Dimethylformamide	80	30
Acetonitrile	60	20
Sodium acetate in water	60	30

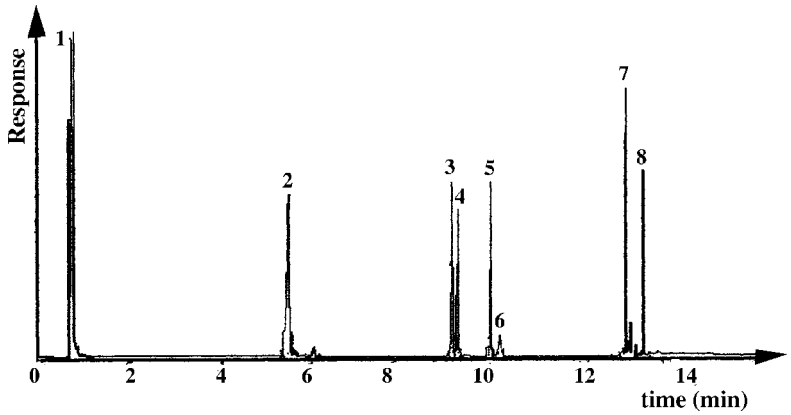

Fig. 1. Chromatogram using hydroxylamine hydrochloride in pyridine and BSTFA. Peaks: 1, solvent; 2, arabinose + xylose; 3 and 4, fructose; 5 and 6, glucose; 7, salicin (internal standard); 8, sucrose. For chromatographic conditions see Section 2.

pyridine and acetonitrile require 80 and 60 °C, respectively. Acetonitrile also required solvent evaporation under nitrogen flow before silylation, increasing total analysis time.

3.3. TMS oximes stability and quantitativity

Quantitative formation was evaluated by comparing relative response factors using salicin as internal standard, results are shown in Table 3. In general reaction yield was lower with acetonitrile, aniline and pyridine gave comparable results.

Stability was evaluated, by running triplicates stored at room temperature versus three replicates stored at 4 °C, each sample replicate was injected three times.

Table 2
Optimum conditions for silylation reaction

Solvent	BSTFA (ml)	Reaction temperature (°C)	Reaction time (min)
Pyridine	0.3	80	10
Aniline	0.3	Ambient (23 °C)	10
Acetonitrile	0.4	60	10

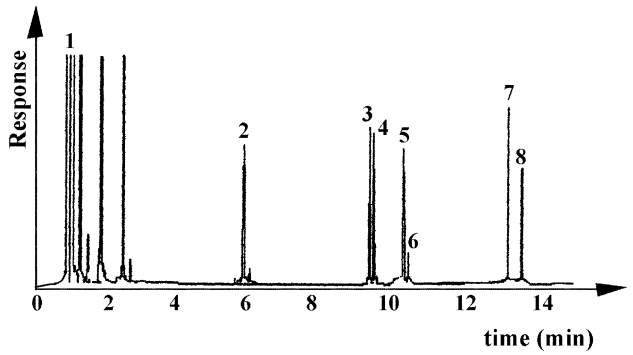

Fig. 2. Chromatogram using hydroxylamine hydrochloride in aniline as solvent and BSTFA as silylation reagent. Peaks: 1, solvent; 2, arabinose + xylose; 3 and 4, fructose; 5 and 6, glucose; 7, salicin (internal standard); 8, sucrose. For chromatographic conditions see Section 2.

Table 4 shows recoveries and R.S.D. for samples stored at room temperature, from these results we can conclude that Oxi-TMS quickly deteriorate in acetonitrile, after a few hours less than 84% of original arabinose + xylose remains in sample and the R.S.D. goes up to 8.8%. On the other hand, pyridine and aniline both stabilize products. This is especially true with aniline, where again arabinose + xylose are the worst case, 96% of the original amount remains in sample with a R.S.D. of 5.5%, other compounds are far more stable and reproducible in aniline.

3.4. Applicability

The optimized method was tested with both fresh pear juice and a processed one, resulting chromatograms are shown in Fig. 3, where the expected Oxi-TMS aldoses are

Table 3
Chromatographic relative response factors for Oxi-TMS aldoses, using salicin as internal standard

Solvent	Response factor			
	Arabinose + xylose	Fructose	Glucose	Sucrose
Pyridine	2.322	1.877	2.235	1.591
Aniline	2.397	5.265	1.387	1.045
Acetonitrile	1.204	1.523	1.587	1.108
Sodium acetate in water–acetonitrile	2.009	2.704	2.380	0.835

Table 4
Stability of Oxi-TMS derivatives

Solvent	Percent remaining (R.S.D., %)			
	Arabinose + xylose	Fructose	Glucose	Sucrose
Pyridine	96 (4.1)	101 (1.6)	91 (7.1)	99 (7.5)
Aniline	96 (5.5)	104 (3.6)	101 (1.4)	100 (2.1)
Acetonitrile	84 (8.8)	91 (8.0)	99 (2.4)	100 (4.5)

Remaining percent for each compound in samples stored at room temperature is shown, with relative standard deviation in parenthesis.

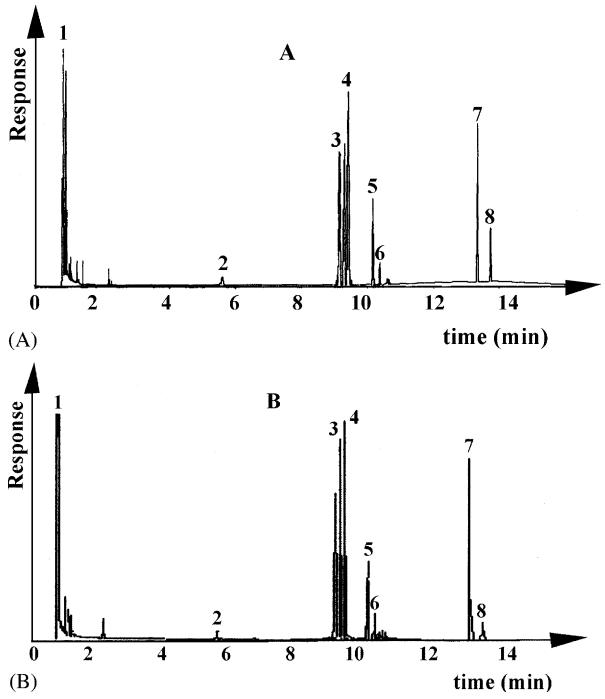

Fig. 3. Gas chromatogram of the TMS oxime sugar derivatives obtained for (A) natural juice and (B) commercial juice, using aniline and BSTFA. Peaks: 1, solvent; 2, arabinose + xylose; 3 and 4, fructose; 5 and 6, glucose; 7, salicin (internal standard); 8, sucrose. For chromatographic conditions see Section 2.

observed, and no by-products are obtained in these chromatograms.

4. Conclusions

Common methods for sugar derivatization use pyridine as solvent for oxime formation; substitution of this compound with aniline reduces analysis time, silylation can be performed at room temperature and more stable products are obtained.

Other authors have proposed acetonitrile, but this solvent requires an extra dehydration step, increasing analysis time and decreasing reproducibility. Also product stability becomes a matter of concern with this solvent.

For silylation, BSTFA proved to be the best reagent, decreasing considerably the number of by-products and requiring a short reaction time.

The method of choice is applicable to processed foods, such as juices and fruits concentrates, affording reproducible results as compared with other literature procedures.

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