Journal of Chromatography, 607 (1992) 191–198
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 139

Post-column derivatization of carbohydrates with ethanolamine–boric acid prior to their detection by high-performance liquid chromatography

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ABSTRACT

The influence of experimental variables such as the wavelength, composition of the fluorogenic reagent, reaction time, reaction and measurement temperatures, and concentration on the post-column derivatization of carbohydrates by reaction with an ethanolamine–boric acid mixture was studied. In order to enhance the signals given by di- and trisaccharides, they were hydrolysed, after elution, with *p*-toluenesulphonic acid, which also provided lower detection limits for monosaccharides. The procedure was applied to the determination of eleven carbohydrates in wines.

INTRODUCTION

Accurate determinations of individual sugars in foods and beverages are becoming more important every day because they not only provide compositional information on the samples, but also help to solve nutritional problems, and problems of adulteration, origin, manufacture, etc. Wine analyses involve some interesting aspects related to pentoses. These substances, which occur in grape juice as complex combinations from which they are released during fermentation [1], cannot be fermented by yeasts, so they are transferred to wine unchanged. Hence their analysis can be of great value in determining the grape variety used to produce a given wine. Confronted with the problem of evaluating both pentoses and other sugars [2] after fermentation, high-performance liquid chromatography (HPLC) with ion-exchange columns was chosen from the many types of HPLC to separate and evaluate carbohydrate samples [3] because of the selectivity of this technique. Taking into account the fact that pentose concentrations rarely exceeds 2 g/l in wines, anion exchange of carbohydrate–borate complexes [4] and fluorescence detection after post-column derivatization with ethanolamine-boric acid [5] was selected because of the simplicity, economy and sensitivity of this technique [6,7]. A preliminary application to some standards of pentoses and other carbohydrates revealed significant differences in analytical behaviour, so a more detailed study of the variables affecting the derivatization–detection process was carried out. This paper reports the results obtained in relation to the parameters with the most marked influence on the post-column fluorescence derivatization reaction between carbohydrates and an ethanolamine–boric acid mixture. The optimized conditions were applied to the analysis of several wines of different origin and manufacture.

EXPERIMENTAL

Reagents

Sugar stardards were purchased from Sigma Al-

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0021-9673/92/$05.00

drich Química (Madrid, Spain). Boric acid, ethanolamine and *p*-toluenesulphonic acid were supplied by Fluka Chemie (Buchs, Switzerland). All other reagents required to prepare buffers and analytical solutions were pro analysis-grade chemicals purchased from Scharlau (Barcelona, Spain).

HPLC apparatus and conditions

The experimental set-up consisted of the following elements: a 6000A dual-piston pump from Waters Assoc. (Milford, MA, USA) that was used to propel the eluent, namely a 0.4 *M* borate buffer at pH 9.35, at a flow-rate of 1 ml/min; a Rheodyne 7125 injector with a fixed-volume (20 μl) loop. The separation was carried out on a glass chromatographic column of 50 cm × 0.3 cm I.D. that was packed with Aminex A-25 borate ion-exchange resin and wrapped in a water jacket. The working temperature was 69°C. Alternatively, a metal chassis of 30 cm × 0.46 cm I.D. packed with the same stationary phase which was customized by Bio Rad Labs. (Richardson, CA, USA) was used and was maintained at 69°C in an oven from Jones Chromatography (Wales, UK). An M-45 pump, from Waters Assoc., propelled the fluorogenic reagent (an aqueous solution containing 20% ethanolamine and 20% boric acid) at a rate of 0.5 ml min. After the merging point the reactants were conveyed into a PTFE capillary of 30 m × 0.3 mm I.D. which was immersed first in a reaction bath filled with glycerine at 140°C and then (1 m) in thermostated water at 20°C to cool the mixture. An SFM 25 fluorescence detector from Kontron Instruments (Zurich, Switzerland) furnished with a 15-μl flow cell was used. Measurements were made at excitation and emission wavelengths of 400 and 445 nm, respectively.

The hydrolysis involved a third pump (VS minipump from Milton Roy, Riviera Beach, FL, USA) to propel an aqueous solution of 1.6 *M* *p*-toluenesulphonic acid through a PTFE capillary (10 m × 0.3 mm I.D.) immersed in the bath at 140°C. Hydrolysis occurred after the carbohydrates were eluted from the column and before the derivatization reaction.

Reaction parameters

To study the influence of the reaction parameters and then to determine their optimal values, experiments were first carried out in non-continuous mode, and then the results were adapted to the chromatographic system and the chromatographic conditions were also studied.

For the non-continuous mode an LS-5 spectrofluorimeter from Perkin Elmer (Beaconsfield, UK) was used.

Wavelength. This was selected by reacting the different carbohydrates assayed with the fluorogenic reagent and recording the excitation and emission spectra of the products.

Reaction time. Volumes of 3 ml of 0.05% solutions of the carbohydrates were reacted with 5 ml of a 2% solution of the fluorogenic reagent in test tubes that were heated for different times and then allowed to cool before the fluorescence intensity was measured.

Fluorogenic reagent. The optimal composition of the ethanolamine–boric acid mixture was chosen from the intensity of the signal obtained for some carbohydrates (20 μg per 20-μl sample) on reaction with different mixtures containing a fixed amount of ethanolamine (15 g/l) and a concentration of boric acid that varied between 3 and 20 g/l. In order to obtain the proportion of mixed reagent to be used in the fluorogenic solution, contents between 2 and 30% in water were assayed.

Reaction temperature. Samples of 20 μg of each carbohydrate were injected and subjected to temperatures between 100 and 150°C, with no passage through the column. In this way the optimal reaction temperature was determined.

Measurement temperature. The mixture obtained after the reaction was cooled to different temperatures prior to arrival at the detector, to obtain the highest fluorescence response.

Hydrolysis. Different concentrations of *p*-toluenesulphonic acid, between 0.1 and 2 *M*, were assayed. In addition, the flow-rate was varied between 0.1 and 0.5 ml/min.

Internal standard calibration graphs

Melibiose was used as internal standard. Stock solutions of eleven carbohydrates (10 g/l) and melibiose (10 g/l) were prepared in nanopure water. Standard solutions were prepared from stock solutions by sequential dilution with nanopure water.

The calibration curves were calculated by the least-squares method. Peak-area ratios between the carbohydrates and melibiose were used to generate

the least-squares regression lines. Concentrations of carbohydrates in the wine samples were obtained by interpolation from these calibration curves using peak-area ratios obtained from unknown samples. A constant concentration (0.1 g/l) of internal standard was added to each sample prior to the analysis.

Wine sample

The proposed method was applied to the analysis of different wine samples from Castile and Leon winemakers, which belong to three Origin Denominations.

The white wines were from Rueda Origin Denomination, where the *V. vinifera* variety of grape most widely grown is *Verdejo*, native to this region. *Palomino* and *Viura* are other varieties grown to a lesser extent.

Rosé wines (Cigales Origin Denomination) are a mixture of *V. vinifera* varieties: *Tinta del Pais* (red), *Garnacha* (red), *Albillo* (white), *Verdejo* (white) and *Viura* (white).

The red wines were from Ribera del Duero Origin Denomination, where the most widespread *V. vinifera* variety is the *Tinta del Pais*, a variant of the classic fruity *Tempranillo*, usually mixed with *Cabernet, Merlot* or *Garnacha* in a small proportion.

Since in most samples the concentration of some carbohydrates was rather low the samples were concentrated ten-fold.

RESULTS AND DISCUSSION

Chromatographic column

Changing the column made by us to one customized in stainless steel provided better performance, though at the expense of longer retention times (Table I). Neither of the columns solved the problem of overlap between ribose and rhamnose.

Wavelength

In Table II the maximum excitation and emission wavelengths are shown. The optimal wavelengths were 400 and 445 nm, respectively.

Reaction time

Fig. 1 shows a typical curve for glucose. As can be seen, the fluorescence intensity increased markedly with increase in the heating time. In fact, appreciable signals were obtained after 15 min heating; this dictated the length and inner diameter of the reaction coil to be used for the post-column derivatization. In order to avoid too long reaction times, a PTFE capillary, 30 m × 0.3 mm I.D., was chosen and was stitched through a steel net with mesh openings to avoid peak broadening as far as possible [8].

TABLE I

RETENTION TIMES (t_R) OF CARBOHYDRATES IN BOTH COLUMNS

Carbohydrate	Retention time (min)	
	Home-made	Purchased from Bio-Rad
Melibiose	10.5	9.3
Sucrose	14.1	12.9
Maltose	17.4	16.3
Lactose	18.1	17.0
Rhamnose	19.6	18.2
Ribose	19.7	18.2
Mannose	25.9	29.9
Fructose	28.4	32.9
Arabinose	30.1	37.1
Galactose	34.7	43.3
Xylose	38.6	49.4
Glucose	51.0	67.7

Fluorogenic reagent

Fig. 2 shows the variation in the peak area as a function of the boric acid concentration. As can be seen, the maximum signal was obtained at a 1:1 ethanolamine/boric acid ratio. Fig. 3 shows that the increase was different for each carbohydrate, thus 20% concentration of each component was selected

TABLE II

OPTIMAL DETECTION WAVELENGTHS (λ) FOR SOME CARBOHYDRATES AFTER THEIR DERIVATIZATION WITH ETHANOLAMINE–BORIC ACID

Carbohydrate	$\lambda_{excitation}$ (nm)	$\lambda_{emission}$ (nm)
Galactose	397	447
Glucose	398	445
Xylose	403	449
Arabinose	398	453
Fructose	400	446

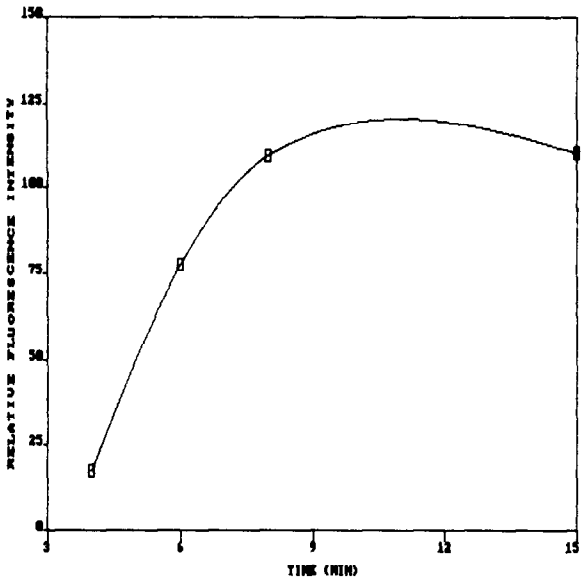

Fig. 1. Relative fluorescence intensity *versus* heating time for glucose.

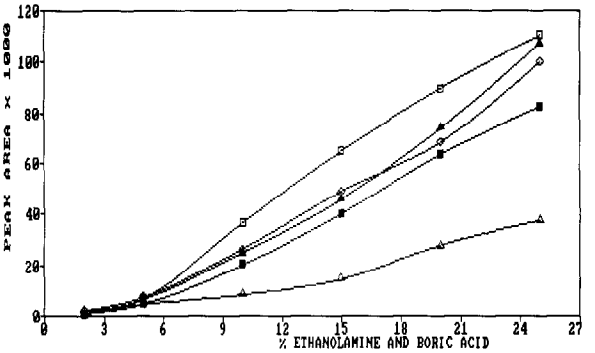

Fig. 3. Peak-area variation for some carbohydrates with the percentage ethanolamine and boric acid (1:1). △ = Lactose; □ = fructose; ◇ = xylose; ▲ = glucose; ■ = galactose.

for subsequent experiments since higher contents increased the mixture viscosity.

Reaction temperature

Figs. 4 and 5 show the results obtained. The peak area varies dissimilarly with temperature for each carbohydrate. As a rule, the maximum signals were obtained at about 140°C for monosaccharides and

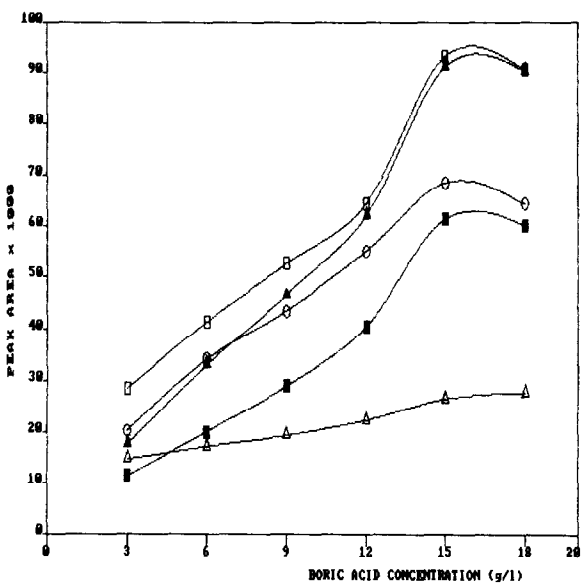

Fig. 2. Influence of boric acid concentration on the peak areas for different carbohydrates at a fixed ethanolamine content. △ = Lactose; □ = fructose; ○ = xylose; ▲ = glucose; ■ = galactose.

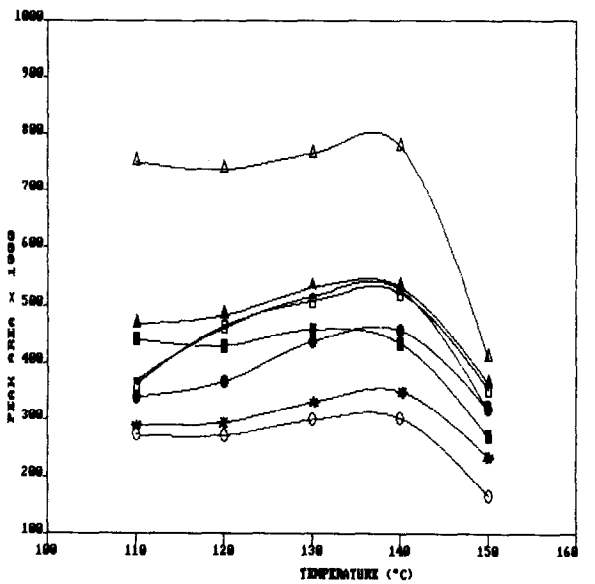

Fig. 4. Temperature influence on the post-column derivatization reaction for the monosaccharides assayed in relation to the peak area. ✳ = Ribose; ▲ = mannose; ■ = galactose; ● = xylose; △ = rhamnose; □ = fructose; ○ = arabinose; • = glucose.

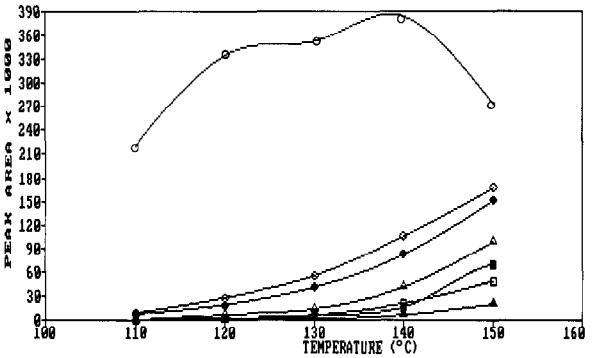

Fig. 5. Temperature influence on the post-column derivatization reaction for di- and trisaccharides assayed in relation to the peak area. ○ = Maltotriose; □ = rafinose; ◇ = lactose; ○ = melibiose; ▲ = melezitose; ■ = sucrose; ◆ = maltose.

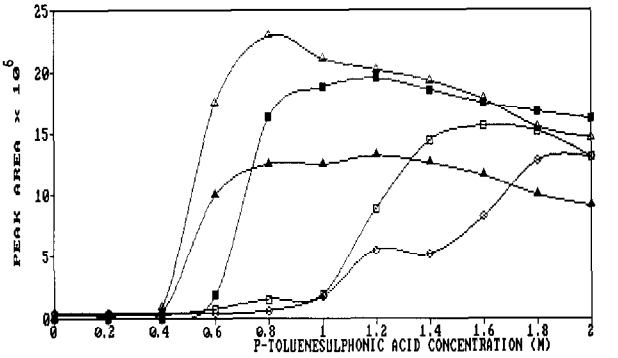

Fig. 7. Peak-area variation of some carbohydrates, with changes of the *p*-toluenesulphonic acid concentration for post-column hydrolysis. △ = Sucrose; ▲ = rafinose; □ = maltose; ■ = melezitose; ◇ = lactose.

occasionally above 150°C for di- and trisaccharides, which obviously featured higher detection limits.

Measurement temperature

As expected, cooling the reaction mixture prior to arrival at the detector resulted in increased fluorescence responses, as can be seen in Fig. 6. However, a temperature of 20°C was chosen for greater experimental convenience and in order to avoid problems in thermostating the flow cell.

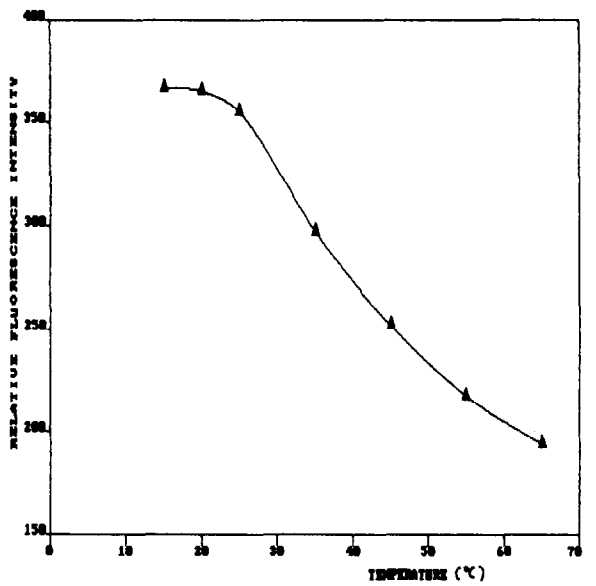

Fig. 6. Variation in relative fluorescence intensity for glucose with changes of measurement temperature.

Hydrolysis with p-toluenesulphonic acid

In Fig. 7 the different signals produced by the carbohydrates (4 μg per 20 μl sample) can be appreciated, but lactose and maltose behaved differently on hydrolysis. An acid concentration of 1.6 *M* was chosen because lactose and maltose signals were quite high and those of the other carbohydrates did not decrease to markedly.

As far as the reagent flow-rate is concerned (Fig. 8), lactose again behaved anomalously. In order to

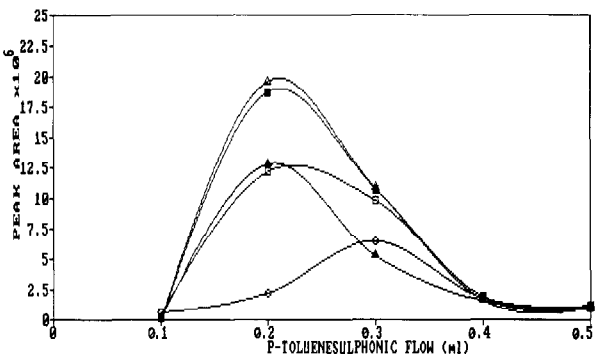

Fig. 8. Relation between the peak area for some carbohydrates and the flow-rate (ml/min) of *p*-toluenesulphonic acid. △ = Sucrose; ▲ = rafinose; □ = maltose; ■ = melezitose; ◇ = lactose.

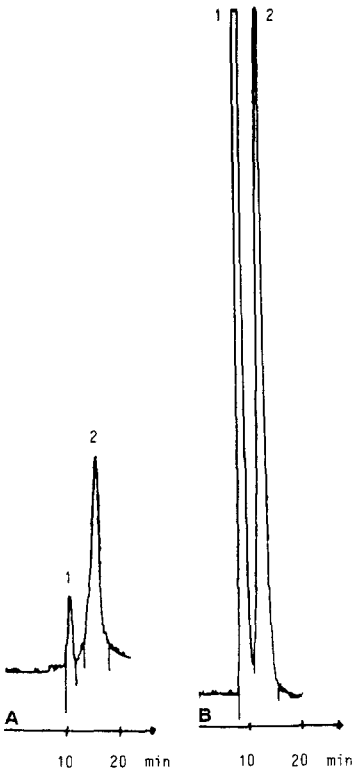

Fig. 9. Chromatogram showing the influence of acid hydrolysis on sucrose and maltose (A) without acid hydrolysis and (B) with acid hydrolysis for the same disaccharide concentration. Peaks: 1 = saccharose; 2 = maltose.

TABLE III

DETECTION LIMITS AND CONCENTRATION LINEARITY RANGES WITHOUT ACID HYDROLYSIS

Carbohydrate	Detection limits (mg/l)	Linearity range (mg/l)
Sucrose	111	100–300
Maltose	20	15–100
Lactose	20	15–100
Ribose	7	5–100
Rhamnose	7	5–100
Mannose	5	2.5–100
Fructose	10	7–100
Arabinose	10	7–100
Galactose	10	7–100
Xylose	8.5	5–100
Glucose	15	10–100

TABLE IV

DETECTION LIMITS AND CONCENTRATION LINEARITY RANGES WITH ACID HYDROLYSIS

Carbohydrate	Detection limit (mg/l)	Linearity range (mg/l)
Sucrose	40	20–200
Maltose	8.5	5–100
Lactose	8	5–100
Ribose	5	2–100
Rhamnose	5	2–100
Mannose	3	2–100
Fructose	6.5	4–100
Arabinose	6.5	4–100
Galactose	6.5	4–100
Xylose	5	2–100
Glucose	10	7–100

obtain the highest possible signal for this sugar an acid flow-rate of 0.3 ml/min was chosen.

Once the optimal acid concentration and flow-rate were selected, it was found by injecting a mix-

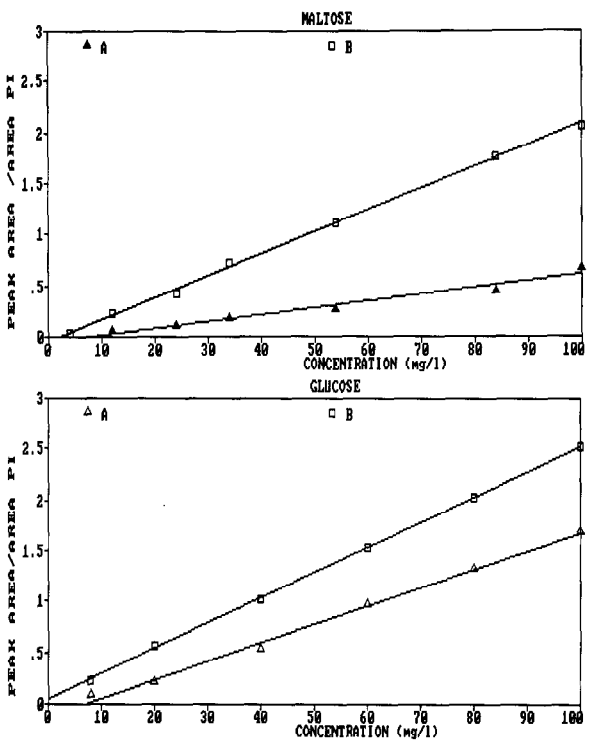

Fig. 10. Standard curves for maltose and glucose (A) without treatment with *p*-toluenesulphonic acid; (B) with treatment.

ture of carbohydrates that hydrolysis improved the signals given by mono-, di- and trisaccharides to such an extent as to make this advisable as pretreatment (Fig. 9).

Standard curves

In Tables III and IV the linearity ranges and detection limits obtained are shown. Fig. 10 shows examples of the standard curves of glucose (monosaccharide) and maltose (disaccharide).

Application

In Table V some of the results obtained are listed as an example. Of the white wines from the same winemaker and vintage, there are differences between V4 (fermented and finished from free-run juice) and V1 (cold settling), V2 (cold settling and treated with activated carbon) and V3 (press juice), indicating that V4 is monovarietal (*Verdejo*) and the others are obtained from a mixture of varieties. In particular, the high levels of fructose and the low levels of galactose are notable.

There are also significant differences between the rosé wine samples from two winemakers and three vintages. For example, from the same winemaker the oldest wine contains xylose, a high ratio of glucose to galactose and a high level of fructose. Comparing R1 and R5 samples (same vintage and different winemaker) reveals great differences in the fructose levels.

In the red wine samples from winemaker 5, note the lack of mannose and xylose and the very different glucose/galactose ratio (<1) in comparison with the results obtained from winemaker 4.

CONCLUSIONS

The use of a post-column fluorescence derivatization reaction between carbohydrates and ethanolamine-boric acid mixtures allows the detection of sugars in complex mixtures previously resolved by HPLC with ion-exchange columns. The reaction is

TABLE V

RESULTS OBTAINED FROM APPLYING THE OPTIMIZED METHOD FOR DIFFERENT WINES

Suc = Sucrose; Mal = maltose; Lac = lactose; Rib = ribose; Rham = rhamnose; Man = mannose; Fru = fructose; Ara = arabinose; Gal = galactose; Xyl = xylose; Glu = glucose.

Sample	Vintage	Carbohydrate concentration (mg/l)									
		Suc	Mal	Lac	Rib/Rham	Man	Fru	Ara	Gal	Xyl	Glu
White wines	*Winemaker 1*										
V1	1990	125	70	46	61	2.5	93	73	204	–	68
V2	1990	109	30	35	54	2.3	122	43	176	–	74
V3	1990	120	20	25	20	–	86	27	123	–	123
V4	1990	127	25	28	39	–	698	–	75	–	208
Rosé wines	*Winemaker 2*										
R1	1980	111	84	48	66	2.3	833	–	164	10	477
R2	1987	173	83	55	63	2.0	270	12	328	–	308
R3	1988	130	41	26	47	2.2	63	–	177	–	89
R4	1989	125	25	25	43	2.5	188	15	228	–	141
	Winemaker 3										
R5	1980	104	62	40	51	2.0	1186	–	227	–	181
Red wines	*Winemaker 4*										
T1	1984	125	102	68	73	6	297	20	109	10	467
T2	1985	127	103	76	95	3	170	60	199	59	221
T3	1986	115	117	79	63	2	160	15	241	–	132
	Winemaker 5										
T4	1984	120	39	26	47	–	106	–	222	–	117
T5	1985	125	60	46	94	–	182	–	316	–	200
T6	1986	111	56	38	64	–	218	–	207	–	143

quite sensitive, yet it is influenced by a number of variables in a continuous system. Thus, the working conditions must be optimized for the carbohydrates to be assayed and variations kept within margins as narrow as possible in order to obtain reliable analytical results.

Hydrolysis with *p*-toluenesulphonic acid before the post-column derivatization reaction markedly enhanced the sensitivity of the detection.

By applying the optimized reactions to wine samples from very similar winemaking practices, origin and vintages, interesting differences between the values for mannose, fructose, arabinose, glucose, xylose and for the glucose/galactose ratio were found.

ACKNOWLEDGEMENT

We thank Iberdrola S.A. (Spain) for financial suport.

REFERENCES

1 W. M. Kiliewer, *Am. J. Enol. Vitic.*, 18 (1967) 33.
2 P. Esau, *Am. J. Enol. Vitic.*, 15 (1967) 187.
3 A. A. Ben-Bassat and E. J. Grushka, *J. Liq. Chromatogr.*, 14 (1991) 1051.
4 J. K. Khym and L. P. Zill, *J. Am. Chem. Soc.*, 74 (1952) 2090.
5 R. Kamada and A. Ono, *Soil. Sci. Plant. Nutr.*, 30 (1984) 145.
6 T. Kato and T. Kinoshita, *Anal. Biochem.*, 106 (1980) 238.
7 V. R. Villanueva, M. Th. le Goff, M. Mardon and F. Moncelon, *J. Chromatogr.*, 393 (1987) 115.
8 B. Lilling and E. Heinz, in J. S. Krull (Editor), *Reaction Detection in Liquid Chromatography (Chromatographic Science Series*, Vol. 34), Marcel Dekker, New York, 1986, p. 27.