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# On-line dialysis with high-performance liquid chromatography for the automated preparation and analysis of sugars and organic acids in foods and beverages ☆

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**Abstract**

A quick, simple and robust technique is described for on-line clean-up and analysis of raw liquid food samples containing complex matrices such as dairy products, soft drinks, and fermented beverages. A completely automated sample preparation system (ASTED XL) provides an efficient way of removing macromolecular and microparticulate interferents by high-performance dialysis, prior to HPLC analysis of the sugars, organic acids and related compounds. Processing samples on-line in the concurrent mode permitted both high reproducibility and optimal throughput.

## 1. Introduction

Determination of sugars and organic acids is of great importance in the food industry. The goals of such analytical work are various, but are mainly oriented towards quality control at all stages of production, from receipt of raw materials, throughout processing, to the final products.

Several HPLC techniques have been proposed to analyze, in most cases separately, sugars and organic acids. Sugars are generally analyzed using an $NH_2$-bonded silica column [1,2]. Organic acids are analyzed using ion-exchange or $C_{18}$ columns [3–5], and recently more specific columns [6]. Detection is mainly by UV or refractive index (RI), when very high sensitivities are not required.

The limiting factor of this type of analysis is sample preparation. Efficient sample clean-up is essential for eliminating high-molecular-mass matrix interferents (e.g. proteins, fats, polysaccharides, very condensed phenolic compounds like tannins), in order not to deteriorate the chromatographic results. However, technological advances in sample preparation have been relatively few, when compared with those accomplished for HPLC. Until recently, the clean-up techniques employed prior to the HPLC determination of sugars and organic acids in liquid food samples have been essentially manual and off-line. These techniques vary from simple filtration to more complex clean-up procedures such as liquid–liquid and solid-phase extraction, precipitation and centrifugation [2,4].

This paper presents a more efficient approach

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to sample clean-up using dialysis to remove the matrix macromolecules, followed by column switching for injection onto the HPLC system. The power of the technique may be further extended, according to the application, by performing a selective enrichment of the dialysate using a small trace enrichment cartridge before injection. The combination of on-line dialysis and trace enrichment provides an efficient and highly reproducible clean-up of raw samples [7–9]. The technique which is completely automated is performed on the ASTED XL (automated sequential trace enrichment of dialysates).

ASTED technology has already been successfully applied in clinical, pharmaceutical, food and environmental fields [8–10]. The on-line clean-up of a variety of raw foodstuffs permitted the determination of colourants in desserts and confectionery, nitrofurans in eggs and meat homogenates, aflatoxins in milk [11–13]. The application describes the use of on-line ASTED clean-up for the development of a simple and robust HPLC procedure for determining sugars, organic acids, and related compounds directly from raw liquid food samples. Such samples include dairy products, soft drinks, and fermented beverages. These samples were chosen for the complexity of their various matrices composed of high-molecular-mass interferents such as proteins, polysaccharides or condensed phenolic compounds.

## 2. Experimental

### 2.1. Chemicals and samples

All chemicals were of analytical grade and the solvents were of HPLC grade. The standards (sugars, organic acids and glycerol) were obtained from Sigma (St. Quentin Fallavier, France). Sulphuric acid was obtained from Merck-Clevenot (Nogent-sur-Marne, France). Water and acetonitrile were obtained from J.T. Baker (Paris, France) and ethanol from Carlo Erba (Rueil-Malmaison, France). All HPLC eluents were filtered before use and degassed with helium. Commercially available food samples were analyzed.

### 2.2. Instrumentation

*ASTED XL*

The sample preparation system was a Gilson ASTED XL unit (Gilson Medical Electronics, Villiers-le-Bel, France), consisting of a large XYZ autosampler, two Model 401C dilutors equipped with 1-ml syringes, one flat-bed dialyser with a donor channel volume of 100 $\mu$l and a recipient channel volume of 175 $\mu$l, fitted with a Cuprophan membrane with a molecular mass cut-off of 15 000, and two automated six-port Model 7010 valves (Rheodyne, Berkeley, CA, USA), one of which was fitted with a 20-$\mu$l injection loop. The system control was performed from a keypad using 722 (version 1.03) software.

*HPLC*

The ASTED XL was coupled to an HPLC system (Gilson Medical Electronics), consisting of a Model 306 pump fitted with a 5SC pump head, a Model 805 manometric module, a Model 831 temperature regulator, a Model 132 refractive index detector, and 715 (V1.2) HPLC system controller software.

Liquid food samples were cleaned-up and analyzed according to the following two methods described in Fig. 1:

Method (a) was used to determine only sugars from soft drinks and dairy products (orange syrup, cola drink, reconstituted baby milk and liquid chocolate yoghurt). In this method the $NH_2$-Zorbax silica column came from Du Pont Co (Paris, France).

In method (b), fruit juices and fermented beverages (grape juice, red wine, white wine, apple juice and cider) were analyzed. The Ion-300 column (Interchim, Montluçon, France) used for simultaneous determination of sugars, organic acids and some related compounds such as glycerol and ethanol, contained a polymeric cation exchanger in the hydrogen form.

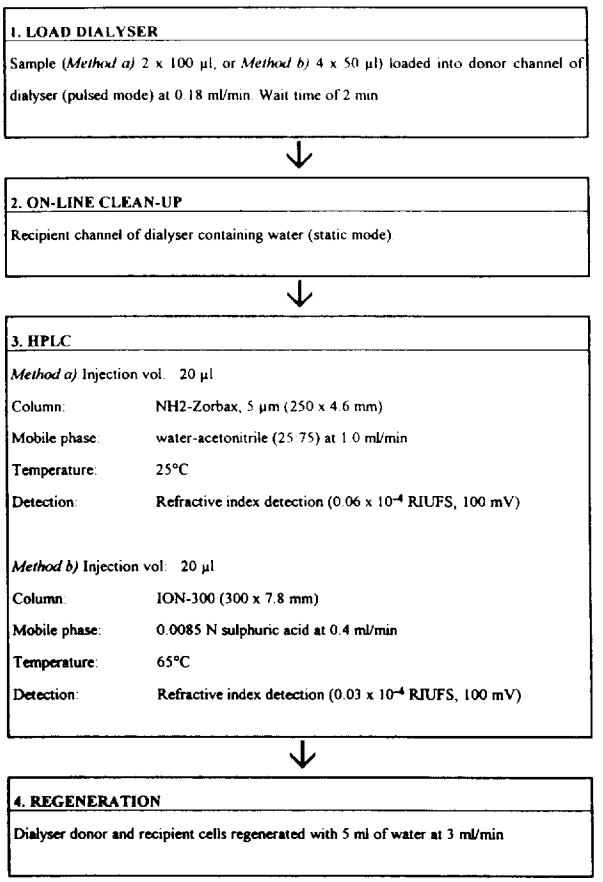


Fig. 1. ASTED XL and HPLC conditions.

## 3. Results and discussion

### 3.1. Optimization of the conditions used in HPLC and ASTED XL, and analytical data

Chromatographic and sample preparation conditions were first optimized using analytical standards diluted in water.

*HPLC*

For both systems, i.e. method (a) and method (b), the main parameters requiring optimization were mobile phase, column temperature and injection volume. In each case, the best compromise had to be established between resolution, analysis time, column lifetime and solvent consumption.

In method (a), the objective was to separate five sugars (i.e. fructose, glucose, sucrose, maltose and lactose). The critical point of the technique described in Fig. 1 was to obtain an acceptable resolution between fructose and glucose within a reasonable analysis time (Fig. 2).

Increasing the percentage of water reduced the retention times of the analytes significantly; a mobile phase of water–acetonitrile (30:70) eluted the five compounds within 10 min, but gave unsatisfactory separation of fructose and glucose ($R_s = 0.4$). On the other hand, a 20:80 composition of this mobile phase provided complete resolution of both analytes, but greatly increased the analysis time, to over 30 min.

The flow-rate was set at 1 ml/min. Higher flow-rates could be used, but with the risks of reducing the resolution, and of decreasing the mechanical resistance of the column, thereby shortening its lifetime.

In this method, the oven temperature was set at 25°C to improve reproducibility of the retention times. Increasing the temperature up to 35°C decreased the analysis time and particularly the retention time of the last peak (lactose) by 9%. Heating the column (to 35°C) was not used, to reduce any stripping of amino groups from the

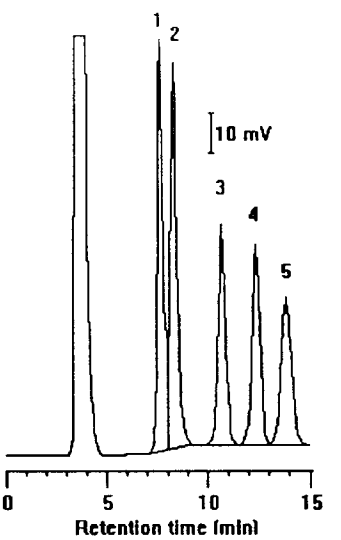


Fig. 2. Chromatogram of standards after processing with ASTED XL. Peaks: 1 = fructose, 2 = glucose, 3 = sucrose, 4 = maltose, 5 = lactose. All concentrations were 10 g/l.

silica surface, to avoid deterioration of the silica itself, and to avoid the formation of Schiff bases between the bonded groups and the reducing sugars [2].

Injection volumes larger than 20 $\mu$l resulted in peak broadening, with a loss of resolution between fructose and glucose.

In method (b), the objective was to separate simultaneously six organic acids (i.e. citric, tartaric, malic, succinic, lactic and acetic acids), two sugars (glucose and fructose), glycerol and ethanol. For this technique, we used ion chromatography with a specific column (Fig. 1), where the primary mechanism for the separation of acids is ion exclusion. Steric exclusion and partitioning are involved in separating other molecules.

Recently, work has been done to optimize the chromatographic conditions using this column for the determination of organic acids, sugars, glycerol and ethanol [6]. The variables to be optimized were the temperature of the column, the concentration of sulphuric acid in the mobile phase, and the flow-rate. In that work, the maximum analysis time was established as 25 min, and the optimum conditions (i.e. temperature 71°C, flow-rate 0.572 ml/min, and concentration of sulphuric acid in the mobile phase 0.005 *M*) determined from theoretical models, gave a satisfactory separation except for glucose, malic acid and fructose, which still had very poor resolution.

Therefore, we modified the above conditions to improve significantly the separation of the three compounds. We decreased the flow-rate, the column temperature, and the sulphuric acid concentration. The variation of the retention times observed were greater for organic acids than for other compounds (the best effects were produced by temperature changes). The chromatogram (Fig. 3) showed a much better separation of the three critical analytes (peaks 3, 4 and 5), but the analysis time was increased to 35 min.

Also with this column, injection volumes greater than 20 $\mu$l resulted in peak broadening and a loss of resolution.

Optimization of the conditions by this method had two objectives: (1) getting the best separation; and (2) preserving the column lifetime by following the manufacturer's recommendations.

*ASTED XL (sample preparation)*

A description of the basic operation of the ASTED XL system is given in Fig. 4. Detection problems do not occur, because in the analysis of food, sugars and organic acids are generally

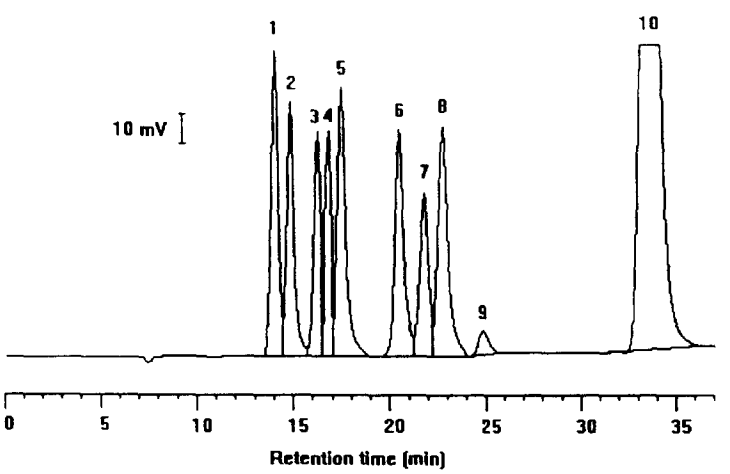


Fig. 3. Chromatogram of standards after processing with ASTED XL. Peaks: 1 = citric acid (6 g/l), 2 = tartaric acid (5 g/l), 3 = glucose (4 g/l), 4 = malic acid (5 g/l), 5 = fructose (5 g/l), 6 = succinic acid (5 g/l), 7 = lactic acid (6 g/l), 8 = glycerol (5 g/l), 9 = acetic acid (1 g/l), 10 = ethanol (121 g/l, i.e. Alc. 15% vol.).

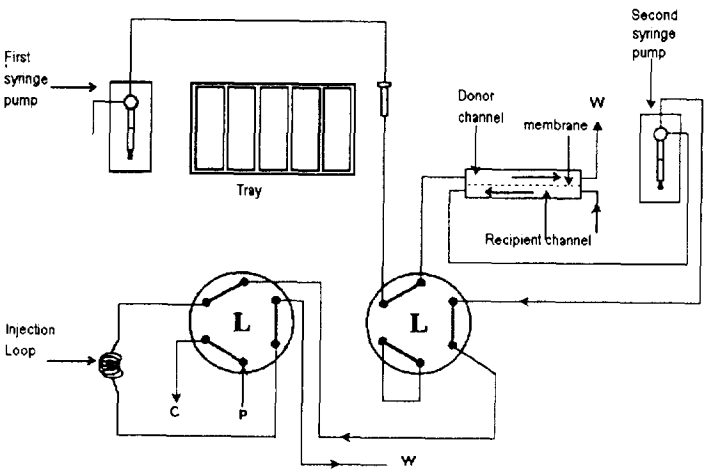


Fig. 4. ASTED XL sample preparation (dialysis and injection process). L = load position; P = HPLC pump; C = chromatography column; W = waste.

present in sufficient quantities. Therefore, on-line dialysis was performed without the need for a trace enrichment step. The sample was loaded into the donor channel of the dialyser and the analytes diffused across the membrane. Then, the dialysate in the recipient channel was transferred into the injection loop. On completion of dialysis, the left injection valve was switched, and the analyte solution was transported by the HPLC mobile phase onto the chromatographic column.

To ensure a favourable concentration gradient across the dialyser membrane, and to optimize the speed and recovery of the dialysis, the liquid flow through both the donor and recipient channels of the dialyser was regulated. Selection of appropriate dialysis models (static, pulsed or continuous) for each channel required consideration of the following points: (1) the absence of a trace enrichment step, which meant that it was not possible to obtain and inject all of the analytes crossing the membrane; (2) the limited size of the injection loop (imposed by chromatographic conditions), which meant that only 20 $\mu$l of the dialysate could be analysed.

Consequently, the pulsed dialysis mode was chosen for the donor channel and the static mode for the recipient channel, at the lowest possible flow-rate (0.18 ml/min) available with the 722 (version 1.03) software, which controlled the instrument.

*Analytical data*

Limits of detection (LOD), recovery rates and linearity were determined for both methods.

In method (a) (Table 1), the LOD was 0.16 g/l for fructose and glucose, and varied from 0.28 g/l to 0.41 g/l for dissacharides. Because of their steric hindrance, recoveries of sucrose, maltose and lactose (4.5–4.9%) were lower than those of the hexoses (6.3–7.1%). Over the linear range the R.S.D.s varied from 2.0% to 9.5%.

In method (b) (Table 2), the LOD varied from 0.04 g/l to 0.11 g/l; the better results observed in this method for glucose and fructose could be explained by a higher sensitivity obtained by using a lower flow-rate inside the flow cell of the detector. Recoveries varied from 6.7% to 9.5% and over the linear range the R.S.D.s varied between 0.6% and 7.9%.

### 3.2. Liquid food sample analysis

In this part of the study, we used internal standards to minimize any possible fluctuations during dialysis caused by the composition of the matrix.

Table 1
Analytical data on the automated determination of sugars using on-line dialysis and amino-bonded silica column HPLC

| Analyte | Limit of detection[a] (g/l) | Recovery[b] (%) | Linearity[c] | | | Range of linearity (g/l) | Precision[d] (R.S.D.) (%) |
|---|---|---|---|---|---|---|---|
| | | | Slope | Intercept | $r$ | | |
| Fructose | 0.16 | 6.3–7.0 | 137 475 | 138 907 | 0.999 | 1–100 | 2.4–9.5 |
| Glucose | 0.16 | 6.3–7.1 | 150 194 | 92 921 | 0.999 | 1–100 | 3.0–7.2 |
| Sucrose | 0.35 | 4.5–4.8 | 94 446 | 121 854 | 0.997 | 3–100 | 2.0–7.6 |
| Maltose | 0.28 | 4.5–4.7 | 77 896 | 140 456 | 0.994 | 3–100 | 3.1–9.1 |
| Lactose | 0.41 | 4.6–4.9 | 66 103 | 140 145 | 0.994 | 3–100 | 2.8–6.3 |

[a] LOD at a signal-to-noise ratio of 3.
[b] Recoveries obtained with two concentrations for each analyte (5 and 10 g/l).
[c] Calibration data (based on areas) obtained from 715 HPLC system controller software; $r$ = correlation coefficient; 5 data points for fructose and glucose, 4 data points for sucrose, maltose and lactose, 5 replicates for each analyte.
[d] Minimum and maximum R.S.D. over the linear range ($n = 5$).

Table 2
Analytical data on the automated determination of organic acids, sugars and related compounds using on-line dialysis and polymeric ion 300 column HPLC

| Analyte | Limit of detection[a] (g/l) | Recovery[b] (%) | Linearity[c] | | | Range of linearity (g/l) | Precision[d] (R.S.D.) (%) |
|---|---|---|---|---|---|---|---|
| | | | Slope | Intercept | $r$ | | |
| Citric acid | 0.07 | 6.7–7.8 | 106 181 | −15 799 | 0.999 | 0.5–10 | 1.8–4.6 |
| Tartaric acid | 0.07 | 7.6–8.5 | 114 009 | −15 789 | 0.999 | 0.5–10 | 1.7–4.7 |
| Malic acid | 0.07 | 7.6–8.4 | 104 302 | −15 448 | 0.999 | 0.5–10 | 1.7–7.0 |
| Succinic acid | 0.07 | 7.7–9.0 | 101 606 | −14 438 | 0.999 | 0.5–10 | 2.0–4.8 |
| Lactic acid | 0.11 | 6.8–8.8 | 78 198 | −15 015 | 0.999 | 0.5–10 | 2.5–7.1 |
| Acetic acid | 0.11 | 8.5–9.5 | 71 700 | −9457 | 0.999 | 0.5–10 | 1.1–3.5 |
| Glucose | 0.05 | 6.7–7.5 | 79 137 | 214 803 | 0.995 | 0.5–100 | 0.6–4.6 |
| Fructose | 0.04 | 7.0–7.4 | 81 014 | 220 514 | 0.995 | 0.5–100 | 0.7–5.0 |
| Glycerol | 0.04 | 7.9–9.2 | 80 587 | 101 728 | 0.998 | 0.4–60 | 1.1–5.0 |
| Ethanol | 0.10 | 8.1–9.1 | 45 626 | 36 144 | 0.999 | 8.1–121 | 1.5–7.9 |

[a] LOD at a signal-to-noise ratio of 3.
[b] Min.–max. recoveries obtained with 3 concentrations for organic acids (0.5, 1 and 2.5 g/l), 3 for ethanol (0.81, 8.1 and 40.5 g/l), 2 for glucose and fructose (1 and 10 g/l) and 2 for glycerol (0.7 and 7 g/l).
[c] Calibration data (based on areas) obtained from 715 HPLC system controller software, $r$ = correlation coefficient, 5 data points except for ethanol (4 data points) in 5 replicates.
[d] Min.–max. R.S.D. over the linear range ($n = 5$).

*Method (a)–Determination of sugars only*

Internal standards selected according to the analysis were automatically added to samples by the ASTED XL before dialysis. Orange syrup was automatically diluted six-fold with water, and the cola drink was automatically degassed by slowly bubbling air through it, both prior to dialysis. Table 3 summarises the results obtained from four real foodstuffs. The efficiency of sample clean-up is particularly well illustrated in the cases of the highly proteinaceous samples, reconstituted baby milk and liquid chocolate yoghurt. Samples were processed concurrently. This means that each sample was prepared whilst the previous one was being analyzed. The analytical time was 15 min per sample (when lactose

Table 3
Carbohydrate content of 4 foodstuffs, with corresponding R.S.D.

| Sample | Baby milk | | Chocolate yoghurt | | Orange syrup | | Cola drink | |
|---|---|---|---|---|---|---|---|---|
| | g/l | R.S.D.% | g/l | R.S.D.% | g/l | R.S.D.% | g/l | R.S.D.% |
| Fructose | – | – | – | – | 46.4 | 3.4 | 30.1 | 2.5 |
| Glucose | – | – | I.S. | – | 60.2 | 3.8 | 31.5 | 4.4 |
| Sucrose | I.S. | – | 80.0 | 3.4 | 7.8 | 7.6 | 63 | 2.3 |
| Maltose | – | – | – | – | 7.0 | 7.0 | I.S. | – |
| Lactose | 20.9 | 3.5 | 42.6 | 4.7 | I.S. | – | – | – |

$n = 10$.

was present in the dialysate). Therefore (apart from the first sample), the sample preparation time of 9 min did not add to the time required to process each sample. The concentrations of the sugars in Table 3 ranged from 7.0 to 80.0 g/l, and the R.S.D. obtained from 10 analyses ranged from 2.3 to 7.6%.

*Method (b)–Simultaneous determination of sugars, organic acids and related compounds*

This type of determination concerns mainly the analysis of fruit juices, and fermented beverages during their ripening and fermentation processes. Propionic acid has been selected as an internal standard (Fig. 5). Again, concurrent sample processing permitted sample preparation (18 min) to be performed within the analytical run-time (35 min when ethanol is present in the sample). The matrix interferents of these samples, mainly polysaccharides or condensed phenolic compounds like tannins, were removed by the dialysis clean-up. Table 4 summarises the results obtained from five beverages.

The natural organic acids analyzed in the two fruit juices, were citric acid, tartaric acid, malic acid for grape juice, and citric acid, malic acid for apple juice. Malic acid could not be determined in the analysis of the grape juice, because of its low level compared to the high levels of the neighbouring sugars. This problem could be solved (if required) by adjusting the HPLC conditions, in particular by lowering the column temperature, which resulted in a displacement of the malic acid out from the pair of

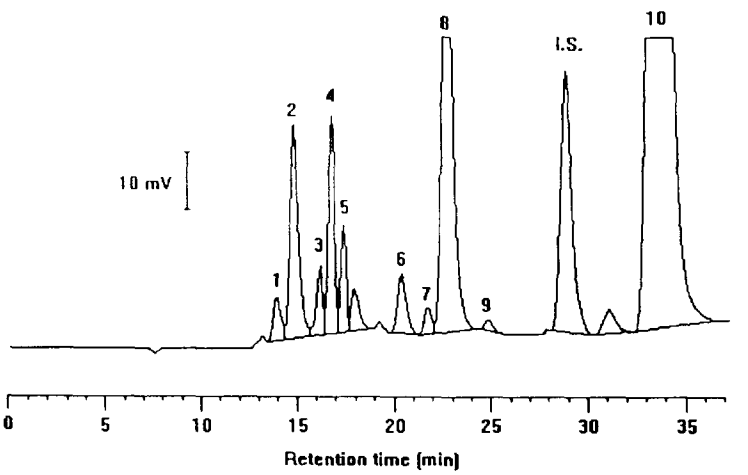


Fig. 5. Chromatogram of a dry white wine after clean-up using ASTED XL. Peaks: 1 = citric acid, 2 = tartaric acid, 3 = glucose, 4 = malic acid, 5 = fructose, 6 = succinic acid, 7 = lactic acid, 8 = glycerol, 9 = acetic acid, I.S. = propionic acid, 10 = ethanol.

Table 4
Organic acid, sugar, glycerol and ethanol content of five beverages, with corresponding R.S.D.

| Sample | Grape juice | | Red wine | | White wine | | Apple juice | | Cider | |
|---|---|---|---|---|---|---|---|---|---|---|
| | g/l | R.S.D.% | g/l | R.S.D.% | g/l | R.S.D.% | g/l | R.S.D.% | g/l | R.S.D.% |
| Citric acid | 0.44 | 2.9 | 0.45 | 4.0 | 0.73 | 7.2 | 0.76 | 3.9 | 0.29 | 7.8 |
| Tartaric acid | 2.22 | 2.9 | 1.99 | 3.4 | 2.64 | 2.5 | – | – | 0.29 | 3.6 |
| Malic acid | n.d. | – | 0.46 | 8.0 | 2.82 | 2.4 | 8.99 | 0.9 | n.d. | – |
| Succinic acid | – | – | 0.70 | 1.6 | 0.71 | 2.0 | – | – | 0.31 | 5.4 |
| Lactic acid | – | – | 1.91 | 1.4 | 0.34 | 1.0 | – | – | 2.81 | 1.1 |
| Acetic acid | – | – | 0.51 | 3.2 | 0.22 | 8.0 | – | – | – | – |
| Glucose | 44.1 | 1.4 | 0.15 | 4.6 | 0.7 | 7.0 | 30.7 | 1.3 | 3.4 | 1.7 |
| Fructose | 39.1 | 1.2 | <LOD | 3.0 | 1.0 | 1.1 | 39.0 | 1.6 | 11.5 | 1.3 |
| Glycerol | 0.9 | 3.0 | 6.6 | 1.0 | 7.4 | 1.4 | – | – | 2.5 | 1.6 |
| Ethanol | 2.2 | 7.8 | 122.0 | 2.2 | 123.0 | 2.0 | – | – | 57.6 | 3.4 |

$n = 5$; n.d. = not determined.

sugars, but with an increase of the HPLC runtime. Another solution could be to use a UV detector with the wavelength set at 210 nm, which would give a much lower signal for sugars.

During alcoholic fermentation, the sugars present in fruit juices are transformed into ethanol by yeasts with production of secondary constituents like succinic acid and glycerol. After alcoholic fermentation, white wines (Fig. 5) do not generally undergo any further changes. However, red wines usually undergo a secondary fermentation process, by bacteria, called "malolactic fermentation", in which the malic acid is transformed into lactic acid. A slight oxidation of the wine may also result in the formation of acetic acid (peak 9). The small peak occurring between the internal standard and peak 10 in Fig. 5 has not been determined. Considering its retention time, it may be methanol, and could come from an enzymatic demethoxylation of some polysaccharides, during the crushing of the fruit and in fermentation. The concentrations of organic acids in Table 4 range from 0.22 to 8.99 g/l, the concentrations of glucose and fructose from below LOD to 44.1 g/l. All R.S.D. values range from 0.9 to 8.0%.

The robustness of the method was studied with a real sample, which was a red wine particularly rich in tannins. We observed a loss of resolution between malic acid and fructose ($R_s = 0.8$) only after 120 injections. After a regeneration of the chromatographic column, according to the manufacturer's recommendations (0.025 *M* sulphuric acid), the initial separation was recovered. We repeated this experiment three times without any other noticeable degradation. Recoveries were unchanged and the dialysis membrane remained efficient.

## 4. Conclusion

This work demonstrates the advantages of on-line sample preparation in the HPLC analysis of liquid food samples. Raw food samples were cleaned up by dialysis and injected directly onto an HPLC system with outstanding reproducibility. The technique was perfectly suited for the removal of macromolecular matrix interferents such as proteins, polysaccharides and condensed phenolic compounds, replacing conventional off-line sample preparation procedures, resulting in more robust HPLC analyses, and widely meeting the criteria required by the food industry for routine analysis. In addition, economical and environmental considerations make this technique very attractive because of the low-cost of consumables (only one membrane for hundreds of analyses) and non-toxic solvent was used (water).

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