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Short Communication

Solid-phase extraction of soluble proteins in grape musts ☆

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ABSTRACT

Samples of soluble proteins were prepared from grape must by dialysis and solid-phase extraction (SPE) for analysis by reversed-phase HPLC. SPE yielded very good recovery rates and saved time as compared with other methods, *e.g.* concentration and purification of proteins from complex matrices such as grape musts.

INTRODUCTION

Considerable improvements have been achieved in solid-phase extraction (SPE) of late. SPE has been used on its own and in combination with conventional methods (centrifugation, filtration, distillation, precipitation, dialysis, etc.) to clean and concentrate samples of many different types for purification and instrumental analysis. Dialysis is a highly suitable method for preparing soluble proteins from grape musts for analysis by electrophoresis or by HPLC. It is simple to carry out, does not denature the proteins, and allows a number of samples to be prepared concurrently, thereby avoiding distortion caused by interference by high- and low-molecular-mass compounds [1,2]. However, the resulting dialysates must be concentrated to adapt protein levels to the sensitivity of the detectors commonly employed.

Concentration by eliminating solvent in a rotary vacuum evaporator (RVE) or by ultrafiltration is slow, and moreover requires pretreatment of samples.

Lyophilization and reversed dialysis do enable several samples to be treated concurrently but do not yield good results in subsequent analysis of the soluble fraction [3].

SPE affords several advantages when concentrating dialysates of this type: it is fast, simple and capable of treating several samples at the same time. In addition, extracts so treated can also be purified by separating out the high-molecular-mass species that are not removed by dialysis itself (clean-up).

Several authors have employed SPE to concentrate or separate proteins from complex matrices, although most such studies have dealt with only one or a small number of proteins [4,5].

The present study compared dialysis and SPE

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in preparing soluble proteins from grape must samples for RP-HPLC analysis.

MATERIALS AND METHODS

Samples

Grapes of the Airén variety collected in Tomelloso (La Mancha, Spain) were used.

Reagents

Deionized water was used in all solutions. Trifluoracetic acid (TFA) was from Fluka (Buchs, Switzerland). Acetonitrile, methanol (HPLC grade) and orthophosphoric acid (analytical grade) were from Panreac (Barcelona, Spain).

Sample preparation

Musts were obtained by lightly pressing the grapes, followed by filtration through a 0.4-μm nylon membrane.

Proteins were separated by dialysis of 150 ml of must against tap water in a Medicell 2 dialysis membrane for 18 h.

Concentration of dialysates

Two procedures were employed to concentrate the dialysates:

(a) An amount of 75 ml of dialysate was concentrated to 2 ml in an RVE. Water was eliminated at low pressure inside the RVE at a temperature of 30°C in order to avoid denaturation and precipitation of the proteins. The concentrate was filtered through a nylon membrane with a pore size of 0.2 μm.

(b) An extraction column (300-mg Extract-Clean RP-18, Alltech, IL, USA) was preconditioned for SPE by first passing 5 ml of methanol followed by 5 ml of distilled water through the column at a flow-rate of 2 ml/min at 20 mmHg (1 mmHg = 133.322 Pa). An 75-ml volume of dialysate was then passed through the column at a flow-rate of 2 ml/min. The bed, undried, was washed first with 2 ml of distilled water and then with 2 ml of a mixture of distilled water and 85% H_3PO_4 (50:50, v/v) at a flow-rate of 1 ml/min using syringe aspiration. Protein elution was carried out by syringe aspiration with 2 ml of methanol at a flow-rate of 2 ml/min.

RP-HPLC analysis

In line with the method previously described by us [2], the linear gradient of acetonitrile in 0.1% TFA–water ranged from 20% to 80% over 45 min. The flow-rate was 1 ml/min.

A 300-Å Nucleosil C_4 150 × 4.6 mm column (Análisis Vínicos, Tomelloso, Spain) was employed to perform the separations.

The injection volume of concentrated extract was 20 μl, and the detector wavelength was 220 nm.

RESULTS AND DISCUSSION

Fig. 1 shows the analysis of RP-HPLC results for the different fractions of Airén grape must obtained by SPE. Chromatogram d, for the methanolic elution fraction, displays considerable reduction of the polyphenolic front as compared with the results obtained by concentrating the dialysate in an RVE [2,6]. This was due to separation of the front compounds into different fractions; some of these compounds were not retained but were carried along with

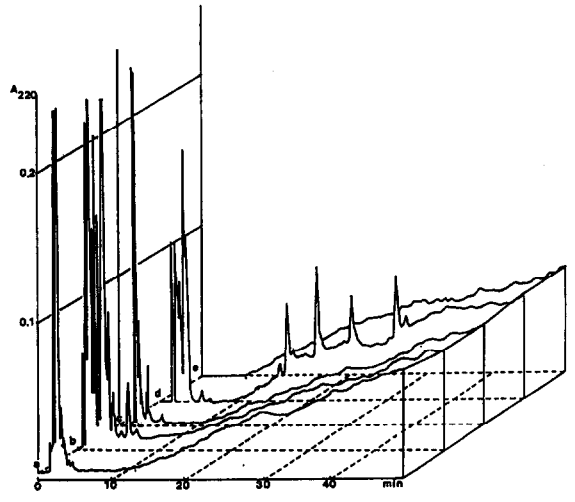

Fig. 1. Chromatogram showing the results of application of RP-HPLC to the different protein fractions of Airén grape must dialysed and concentrated by SPE. (a) Exclusion fraction of the dialysate concentrated 35 times using an RVE. (b) Fraction obtained by washing with distilled water. (c) Fraction obtained by washing with a water–H_3PO_4 mixture (50:50, v/v). (d) Methanolic elution fraction. (e) Methanolic post-elution fraction.

the dialysate, while other compounds eluted during washing (chromatograms b and c).

The reversed-phase SPE columns also acted as precolumns, thereby increasing the working life of the analytical column and enhancing analysis quality.

Elution carried out with 2 ml of methanol, a solvent with high eleutropic power, yielded sufficiently good results for recovery of all the separated proteins (chromatogram d). The appearance of the resulting peaks and the baseline was similar to that obtained for analyses of dialysate concentrated in an RVE [2,6].

Use of the eluting methanolic and acetonitrile fractions in the analyses did not improve recovery. Chromatograms for these analyses were nearly flat (chromatogram e).

The 300-mg extraction columns sufficed to retain the entire protein fraction (2–3 mg). No remaining proteins were detected either in the exclusion fraction of the dialysate afterwards concentrated in an RVE (chromatogram a) or in the washed fractions prior to elution with methanol (chromatograms b and c).

Table I sets out the quantitative data (mean values and relative standard deviations for three replications) for the RP-HPLC analysis and recovery of the soluble proteins from SPE as percentages of the values for the analysis of the RVE-concentrated extract (taken as 100% recovery).

TABLE I

MEAN PERCENTAGE PROTEIN RECOVERY VALUES FOR AIRÉN GRAPE MUST CONCENTRATED BY SPE AND ANALYSED BY RP-HPLC

Magnitude	Protein No.						
	1	2	3	4	5	6	7
t_r(min)	15.1	18.2	21.0	25.0	25.8	27.3	28.2
P_i(%)[a]	20.5	33.3	19.0	1.0	–[b]	19.0	7.1
R.S.D.(%)	6.5	5.0	4.1	7.1		5.5	5.4
R_i(%)[c]	155.3	100.9	87.9	35.0	–	95.2	107.5

[a] Mean percentage value for each protein and R.S.D. calculated for three replications using samples prepared by SPE.

[b] Minor protein (0.9%) determined in the sample concentrated using an RVE [3].

[c] Recovery values for each protein concentrated by SPE as a percentage of the results for samples concentrated using an RVE (100% recovery):

$$R_i(\%) = \frac{\text{Area}_i(\text{SPE})}{\text{Area}_i(\text{RVE})} \times 100$$

On the whole, the differences between the two methods were slight, although higher recoveries were achieved with SPE, which yielded a weighted mean of $R = 108.2\%$[1].

The most significant differences were recorded for the higher recovery (R) of protein 1 by SPE (155.3%) and the lower recoveries of minor proteins 4 and 5, once again as percentages in respect of the results obtained for concentration using an RVE.

$$^1R = \frac{\Sigma P_i \times R_i(\%)}{100}$$

Reproducibility of the SPE-based method was very good (relative standard deviations ranged from 4 to 7%).

In terms of performance, SPE enabled individual samples to be prepared in 30 min, a saving of one-third of the amount of time required when an RVE was used to concentrate the dialysates. Moreover, the method presented the further advantage of being able to process 24 samples simultaneously using commercially available systems (vacuum manifold, Alltech).

SPE makes it possible to automate the entire procedure by incorporating programmable equipment and an automatic injector and thus adapt it for on-line applications. This is an extremely important advantage, because it enables the method to be employed routinely in soluble protein analysis and in producing reliable determinations of the origin of grape musts [2,7] by reducing analysis time and automating sample processing.

CONCLUSIONS

Very good recovery rates were achieved when SPE was utilized to concentrate the soluble proteins in grape musts. Methanol proved to be suitable solvent for elution of the proteins for subsequent analysis by RP-HPLC.

SPE achieved good purification of protein extracts while reducing the time needed for

sample processing, thereby facilitating the application of this method.

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