ELSEVIER

Food Chemistry 83 (2003) 507–513

Food Chemistry

www.elsevier.com/locate/foodchem

Fractionation and characterization of soluble proteins from cider☆

Domingo Blanco Gomis*, Yoana Expósito Cimadevilla, Sara Junco Corujedo, Mª Dolores Gutiérrez Álvarez

Departamento de Química Física y Analítica, Facultad de Química, Universidad de Oviedo, Julián Clavería 8, E-33006 Oviedo, Asturias, Spain

Received 1 November 2002; received in revised form 21 February 2003; accepted 13 March 2003

Abstract

Soluble protein characterization of cider was carried out by reversed-phase high performance liquid chromatography (RP-HPLC) and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). RP-HPLC protein separations by means of their hydrophobicity were performed on a C_{18} (250×4.6 mm i.d., 5 μm, 300 Å) column, using a mobile phase of acetonitrile-acidified water in gradient mode. The protein identifications were carried out by UV spectra analyses. The SDS–PAGE patterns showed several bands with molecular weights between 20 000 and 97 000 daltons. Several methodologies were tested to isolate and preconcentrate the cider proteins prior to their separation and detection. The best results were obtained using dialysis to remove low molecular mass contaminants followed by lyophilization to concentrate the proteins.

Keywords: Protein; Cider; RP-HPLC; SDS–PAGE

1. Introduction

Cider is a sparkling, weakly alcoholic beverage, closely linked to the history of Asturias (Spain) and other countries such as France or England. This beverage is manufactured by traditional methods, namely slow pressing, spontaneous clarification and fermentation and bottling without stabilization. As a sparkling drink, the characteristics of the foam are of great importance, as occurs with sparkling wines, beer or other sparkling beverages. The foam is known to be influenced by fruit variety, harvest and the technological processes employed in making sparkling beverages, since all these factors affect their chemical composition. Several studies have been carried out to ascertain what compounds influence the foam. Pueyo, Martín-Alvarez, and Polo (1995) found that polysaccharides, fatty acids and proteins contribute to foam formation and stability in wines. As can be seen, most of these compounds exhibit surfactant properties.

Studies carried out on base wines used for making sparkling wines (Andrés-Lacueva, López-Tamames, Lamuela-Raventós, Buxaderas, & De la Torre-Boronat, 1996; Brissonnet & Maujean, 1993; Robillard, Despuech, Viaux, Malvy, Vignes-Adler, & Duteurtre, 1993) have shown the existence of a relationship between protein concentration and foam quality. According to this study, the proteins are attracted by the CO_2 bubbles that appear during this fermentation process. This results in stability of the bubbles and the appearance of foam. For this reason, it is necessary to characterize these proteins. In particular, it will be useful to know their isoelectric points, their molecular weight and their hydrophobic characters to determine which of these parameters are important in foam constitution.

However, in several studies realized by Brissonnet and Maujean (1993), no differences were detected in molecular weight or in isoelectric point between the wine foam and the remainder of the wine. These parameters, therefore, do not seem to have much importance. The aforementioned authors show by hydrophobic interaction chromatography that hydrophobic proteins contribute more to foam constitution than their hydrophilic counterparts.

Due to the structural complexity of proteins and the great variability that exists, the use of modern analytical techniques is required for their analysis. The majority of

☆ This research was conducted at the Departamento de Química Física y Analítica, Facultad de Química, Universidad de Oviedo, Julián Clavería 8, E-33006 Oviedo, Asturias, Spain.

* Corresponding author. Tel.: +34-985-103490; fax: +34-985-103125.

E-mail address: dbg@sauron.quimica.uniovi.es (D.B. Gomis).

0308-8146/03/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/S0308-8146(03)00132-8

studies on food and fermented beverages have been carried out using chromatographic methods such as reversed-phase high performance liquid chromatography (RP-HPLC) (Bobe, Beitz, Freeman, & Lindberg, 1998; González-Lara & González, 1991; González & González-Lara, 1993; Knuutinen & Harjula, 1998; Santoro, 1995; Trujillo, Casals, & Guamis, 2000) or hydrophobic interaction high performance liquid chromatography (HI-HPLC) (Brissonnet & Maujean, 1993; González-Lara & González, 1996), which has less risk of protein denaturation compared to RP-HPLC, but entails the difficulty of analyzing the results as well as interference by hydrophobic contaminants. Fast protein liquid chromatography (FPLC; Dorrestein, Ferreira, Laureano, & Teixeira, 1995; Luguera, Moreno-Arribas, Pueyo, Bartolomé, & Polo, 1998) was found to be a highly suitable technique for the study of grape must and wine proteins.

Nowadays, these techniques compete with the more traditional SDS–PAGE habitually employed in these types of studies (Luguera et al., 1998; Santoro, 1995; Yokotsuka, Nozaky, & Takayanagi, 1994).

Wall, Tait, Eastwell, Reid, and Beveridge (1996) analysed proteins in varietally derived apples juices by SDS–PAGE, finding the presence of a protein of approximately 32 000 daltons in some juices. They discussed the possible relationship of this protein to haze formation in clarified apple juice. However, no procedures have been described in the literature to characterize these compounds in cider.

On the other hand, separation and characterization of the proteins in fermented beverages is a difficult task, as the proteins are minor constituents and they have a strong association with polyphenols and other non-protein compounds. The mechanism of association with the former appears to be a non covalent interaction in which protein molecules are held together by polyphenol compounds acting as bridge (Siebert, Troukhanova, & Lyunn, 1996) and this joining has been proposed as the primary cause of haze formation in fruit juices or others beverages (Boye, 1999; Siebert, Carrasco, & Lyunn, 1996). For this reason, the protein analysis is usually preceded by a purification step.

Dialysis is a highly suitable method for preparing soluble proteins from fermented drinks for analysis by electrophoresis (Luguera, Moreno-Arribas, Pueyo, & Polo, 1997; Polo, Cáceres, Palop, Dizy, Pueyo, & Martín-Alvarez, 1989; Santoro, 1995) or by HPLC (Brissonnet & Maujean, 1993; González & González-Lara, 1993; González-Lara & González, 1996; Polo et al., 1989; Santoro, 1995). It is simple to carry out, does not denature the proteins and allows a number of samples to be prepared concurrently. However, the resulting dialysates must be concentrated to adapt protein levels to the sensitivity of the detectors commonly employed. The different techniques employed in dialysate concentration are solvent elimination in a rotary vacuum evaporator (RVE; González-Lara & González, 1991; González & González-Lara, 1993), lyophilization (Luguera et al., 1997; Polo et al., 1989; Santoro, 1995), solid-phase extraction (SPE; González & González-Lara, 1993) and concentration by introducing a dialysis membrane in a polyethylene glycol (PEG) solution (Brissonnet & Maujean, 1993; González-Lara, Correa, Polo, Martín-Alvarez, & Ramos, 1989; González-Lara, Polo, Correa, & Ramos, 1989). Other authors have employed precipitation to concentrate or separate proteins from complex matrices (Santoro, 1995; Waters, Wallage, & Williams, 1991; Yokotsuka et al., 1994).

This paper is specifically concerned with the application of the HPLC method to the characterization of soluble proteins from cider. Dialysis procedures followed by several protocols of sample concentration were tested to isolate and concentrate proteins. Furthermore, protein assessment was carried out by SDS–PAGE to confirm their presence in the cider sample and to determine their molecular weights.

2. Materials and methods

2.1. HPLC equipment and conditions

HPLC analyses were performed on a Shimadzu HPLC system (Columbia, MD, USA) equipped with two LC-10 AD pumps, a UV–vis SPD-M10AD photodiode array detector, a Sil-10 AD automatic injector and a Gastor 150 LCD on-line degasificator.

A Supelcosil C_{18} column [Teknokroma (Barcelona, Spain), 250×4.6 mm i.d., 5 μm, with a pore size of 300 Å] and a Nucleosil C_4 column (Análisis Vínicos (Tomelloso, Spain), 150×4.6 mm i.d., 5 μm, with a pore size of 300 Å) were used. In both cases, a guard column was inserted to protect the analytical column. To study the behaviour of these columns, standards of lysozime, trypsin inhibitor, carbonic anhydrase and albumin chicken egg (ovalbumin) (Sigma, St. Louis, MO, USA) were selected; Fig. 1 shows the results obtained employing the same concentration for each one and the chromatographic conditions mentioned earlier. A substantial improvement in the separation of standards was obtained using a C_{18} column.

The analysis of the standards and cider samples was monitored at 220 nm and the absorption spectra of compounds were recorded between 190 and 370 nm. The mobile phase was water (high purity water, obtained through a Millipore Milli-Q system, Milford, MA, USA) with 0.1% trifluoroacetic acid (Romil, Loughborough, UK), eluent A and water–acetonitrile (HPLC-grade, Romil) 5:95 (v/v) with 0.1% trifluoroacetic acid, eluent B. The chromatographic experiments were carried out at a flow rate of 1 ml/min

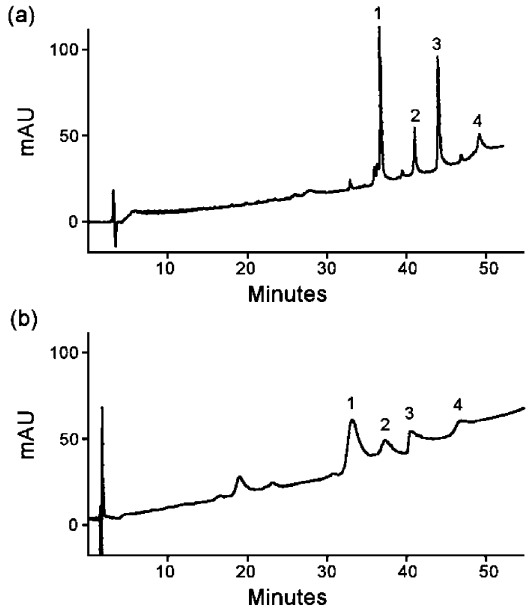

Fig. 1. Protein standards separation on: (a) C_{18} column (250×4.6 mm i.d., 5 μm, 300 Å) and (b) C_4 column (150×4.6 mm i.d., 5 μm, 300 Å). (1) Lysozime, (2) trypsin inhibitor, (3) carbonic anhydrase and (4) ovalbumin. UV detection at 220 nm.

and a temperature of 38 °C in gradient mode. The eluent B percentage thus ranged linearly from an initial value of 0% to a final value of 50% for 45 min. After each run, the column was washed with 100% B for 10 min and equilibrated with 100% A for 15 min. All mobile phase solutions were filtered through a 0.45-μm membrane filter. The injection volume of protein extract was 50 μl. Prior to injection, samples were filtered through PVDF Durapore® (Millipore) 0.45-μm filters. Identification of sample compounds was carried out by spectral analysis.

2.2. Sample preparation

Natural cider was sonicated for 5 min to free the proteins and centrifuged at 3000×*g* for 30 min to eliminate solid substances that could interfere in the analysis. The proteins were isolated by dialyzing (dialysis membranes Spectra/Por® Molecular Weight Cut Off: 3500 daltons, Spectrum, Rancho Domínguez, CA, USA) the cider against ultra pure water at 4 °C, changing the water three times a day for two days.

Four alternative procedures, schematically represented in Fig. 2, were employed to concentrate the dialysate sample. In the first procedure, proteins purification and concentration was achieved by solid–liquid extraction (visiprep Vacuum manifold, Supelco, Bellefonte, PA and Vacuumbrand GmbH pump, Werthein, Germany) using a C_{18} cartridge (Sep-PAK®, 1000 mg, Varian, Harbor City, CA, USA) previously conditioned by passing 5 mL of methanol (Romil) followed by 5 ml of water. A 100 ml of dialysate was passed through the cartridge and the bed was washed with 2 ml of water and subsequently with 2 ml of water with 85% H_3PO_4 (50:50, v/v). Protein elution was carried out with 2 ml of methanol and the solution was filtered and injected into the chromatographic system.

The second concentration procedure was carried out by introducing a dialysis bag in a polyethylene glycol (Sigma) solution 30%. The remaining solution was centrifuged at 3000×*g* for 10 min and finally concentrated in a rotary vacuum evaporator (Heidolph, Schwabach, Germany) at 30 °C. The sample was recovered in 1 ml of water and was injected after prior filtration through a 0.45 μm PVDF syringe filter. In this way, the sample was concentrated 50 times.

An alternative to the above procedure was to concentrate the sample directly in the RVE. Thus, 50 ml of cider, including or not including a filtration step through a PVDF Durapore® membrane with a pore size of 0.22 μm, was dialysed and subsequently concentrated to 1 ml in a 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.

Finally, proteins concentration was carried out by lyophilization (Virtis lyophilizator, New York, USA) with or without prior filtration. The lyophilizate was dissolved in 1 ml of water and the sample was filtered and injected in the chromatographic system, as in the aforementioned procedures.

2.3. SDS–PAGE analysis

Laemli's method (Laemli's, 1970) for discontinuous electrophoresis was performed in a Power N Pac 300 electrophoresis instrument from Bio-Rad (Hercules, CA, USA) [T(acrylamide plus bisacrylamide, w/v) = 12.3%; C (bisacrylamide:acrylamide, plus bisacrylamide, w/v) = 2.6%, resolving gel (T = 4%, C = 2.6%, stacking gel)] to estimate the presence and approximate number of proteins when the purification and concentration stages of samples were finished. Electrophoresis was carried out on plates of 100 mm×82 mm×1 mm at room temperature at a constant current setting of 250 mA for approximately 1 h. Bio-Rad SDS–PAGE Molecular Weight Standards, range from 21 500 to 200 000 daltons were used as a marker.

The proteins bands were visualized by silver staining (ICN). This technique had also served to characterize proteins by means of their molecular weight.

3. Results and discussion

In order to separate proteins in the cider samples, we chose a Supelcosil ODS (250×4.6 mm i.d., 5 μm, with a pore size of 300 Å) and a mobile phase made up of ultra

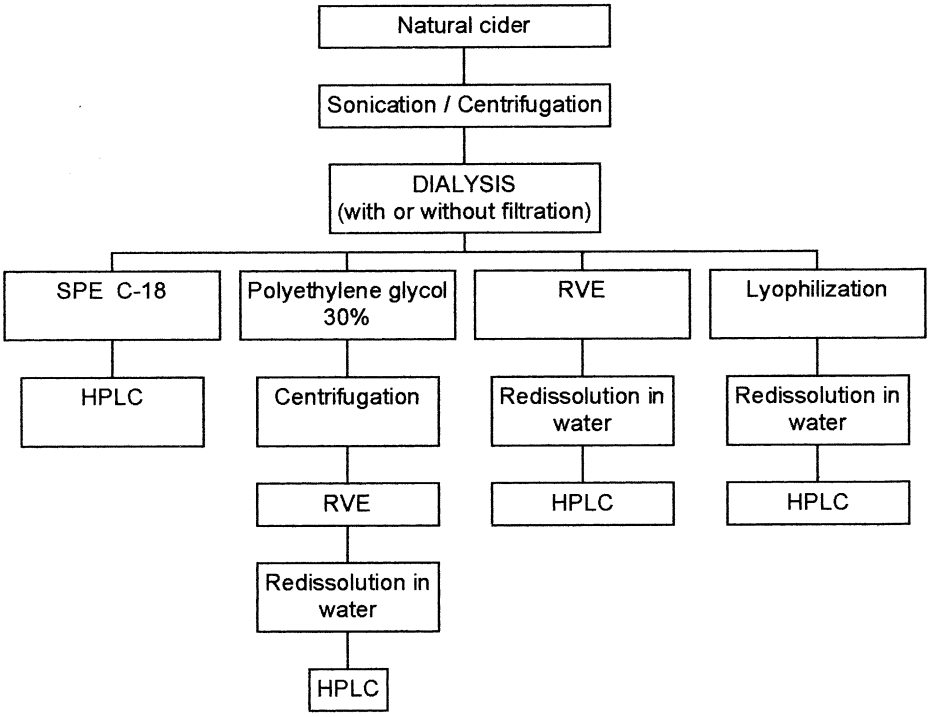

Fig. 2. Schematic diagram of sample preconcentration methods. (RVE) rotary vacuum evaporator; (SPE) solid phase extraction.

pure water, acetonitrile and trifluoroacetic acid as ion pairing, whose addition had the effect of decreasing the retention time of a protein sample and of improving peak shapes. A possible explanation for the effect of ion-pairing reagents on the retention of proteins on a reversed phase column can then be based on the fact that the non-polar area of the protein molecule, which is available for interaction with the reversed phase, is substantially reduced (Hancock & Sparrow, 1983).

Purification and concentration of the cider samples was necessary to analyse proteins by HPLC or SDS–PAGE. Different cleaning techniques were studied, namely precipitation, dialysis, ionic exchange solid–liquid extraction. Dialysis is a simple technique and, in this case, offers better results than the others, but a concentration step of the dialysate was necessary. For this reason, four concentration procedures were assayed, as can be seen in Fig. 2. The first of these includes a solid–liquid extraction using C_{18} cartridges, a significant loss of proteins being observed. This procedure was accordingly rejected.

When the treatment includes a concentration step with polyethylene glycol, the chromatogram obtained shows an interesting protein profile with 11 main peaks and many other minority peaks. As can be seen in Fig. 3a, an important number of proteins elute early on, after 12 min of the chromatogram, which could be in agreement with the predictable, greater presence of less hydrophobic proteins in the cider samples. The baseline improves markedly when the cider sample was filtered through a 0.22 μm PVDF membrane prior to dialysis. However, this treatment was very lengthy, as the first concentration stage with PEG lasted 5 h and did not manage to eliminate all the solvent, so a second concentration step was needed in the RVE. For this reason, the next treatment was carried eliminating the PEG concentration stage. An important baseline drift that makes the detection of proteins difficult was observed in this case. Consequently, as in the previous procedure, it was necessary to filter the sample before dialysis. Fig. 3b shows the chromatogram obtained using this subsequent procedure. Although the baseline improves, a marked loss of proteins was observed.

Finally, preconcentration was carried out by lyophilization. It was again confirmed that sample filtration prior to dialysis improves the sensibility response. Fig. 3c shows the chromatogram obtained including the filtration step. As can be seen, this procedure displayed the best results, since the proteins were visualized better.

The retention times for proteins were similar in all concentration treatments tested. Identification of compounds was carried out by recording their absorption spectra. Typical protein spectra (data not shown) with two maxima at 203 nm due to the peptidic bonds of the protein and 280 nm due to the phenyl groups of the aromatic amino acids of the proteins were observed, particularly in the last peaks of the chromatogram that pertain to the more hydrophobic proteins. However, spectra of the early chromatographic peaks pertaining to less hydrophobic proteins also exhibit relative maxima at 250 and above 300 nm. This suggests that phe-

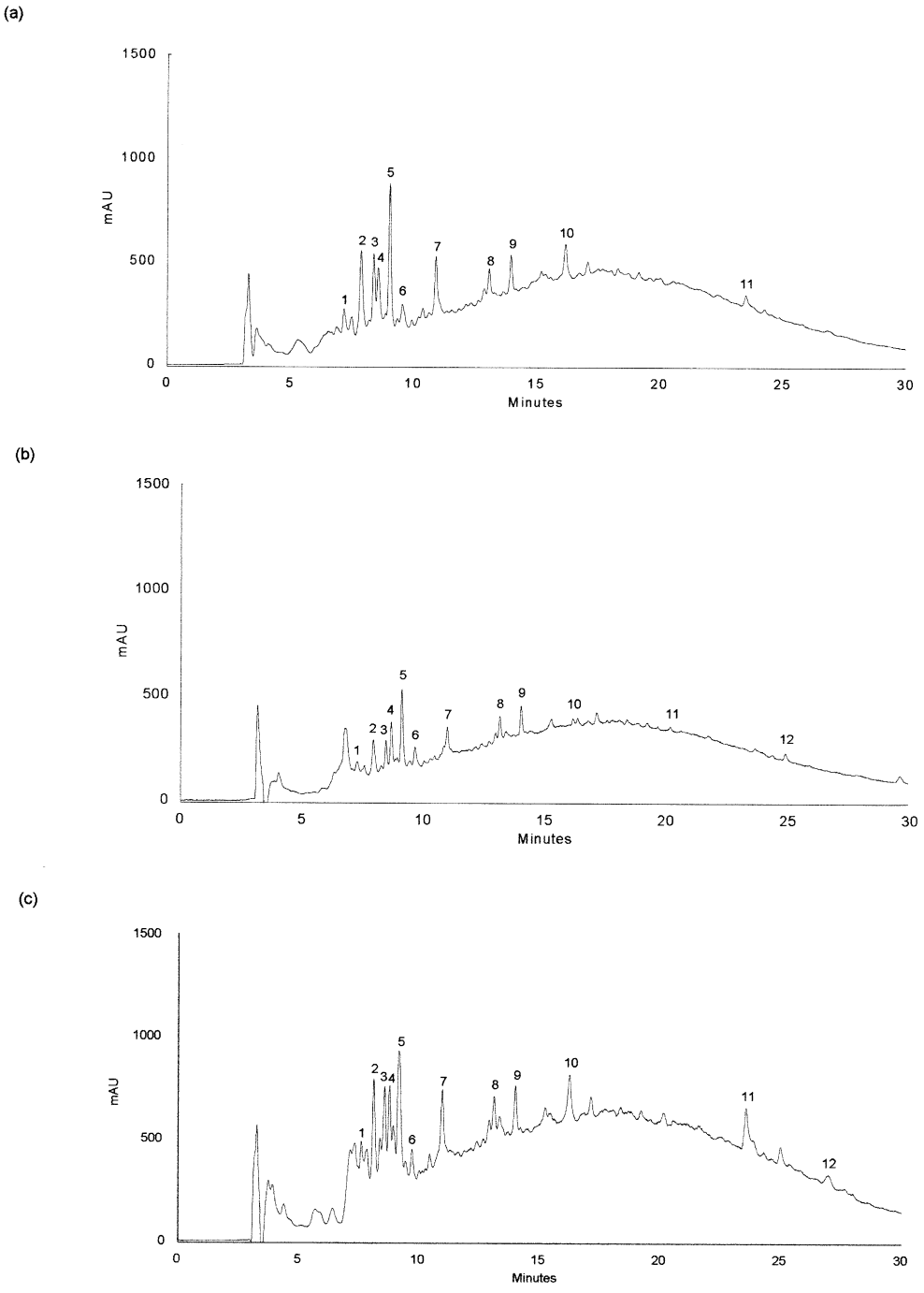

Fig. 3. HPLC profiles of natural cider protein fractions obtained by means of different procedures: (a) dialysis and concentration with polyethylene glycol; (b) dialysis and concentration by RVE; (c) dialysis and concentration by lyophilization. Conditions as Fig. 1a.

nolic compounds might be present forming complex phenolic polymers associated with proteins, as was reported in sparkling wines (Luguera et al., 1998).

Table 1 shows the retention times, the area percentages and the precision of each protein fraction calculated in the last methodology, tested with the same cider. The results were obtained by these replicates. As can be seen, the less hydrophobic proteins accumulate the major area percentage. Effectively, the seven early peaks, eluted in less than 12 min, accumulated more than 70% of total area. The precision in the retention times, is excellent, as Table 1 shows. Regarding the precision in the area percentage of each proteic fractions is reasonably good, except the first fraction ($t_R = 7.6$ min), however it has not a lot of importance since it is not a majority fraction.

Classical electrophoresis in SDS–PAGE was also used to confirm the presence of proteins and their approx-

Table 1
Protein retention time and area percentage values for cider retentates concentrated by lyophilization and analysed by RP-HPLC

Proteic fraction[a]	t_R (min)±RSD (%)	Area (%)±RSD(%)
1	7.6±0.7	4.1±19.9
2	8.1±1.3	16.5±4.7
3	8.5±0.6	9.6±3.8
4	8.7±0.6	6.9±3.7
5	9.1±0.6	23.1±2.3
6	9.7±0.5	3.8±1.4
7	11.0±0.5	7.7±4.0
8	13.1±0.1	2.9±10.0
9	14.0±0.1	4.7±0.8
10	16.2±0.1	8.7±9.9
11	23.5±0.2	8.7±0.2
12	26.9±0.2	3.2±2.5

[a] Calculated as area percentage of each proteic fraction over total area percentage of 12 proteic fractions of one of the chromatograms obtained.

imate number of bands when the process of cleaning and concentration of sample proteins was concluded, thus enabling the characterization of proteins by means of their molecular weights. As can be seen in Fig. 4, in spite of the marked differences in protein detection observed by the chromatographic method, the same bands were observed when the retentate was concentrated by lyophilization or RVE, the intensity also being smaller in this latter case.

As can be observed by comparing the results obtained in the slab gel with the chromatograms, there is one more presence of proteins of low molecular weight, which could be related to the larger presence of less hydrophobic proteins.

Eleven protein fractions are observed when the sample is cleaned by dialysis and concentrated by RVE,

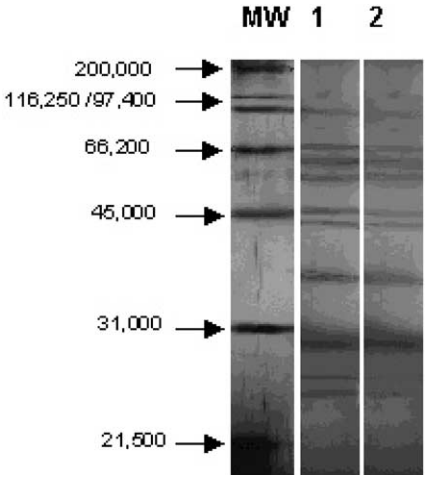

Fig. 4. SDS–PAGE analysis of protein concentrate from natural cider (silver stained). Key: (MW) molecular weight standards in daltons; (1) Protein bands obtained when the cider is dialyzed and concentrated by lyophilization; (2) Protein bands obtained when the cider is dialyzed and concentrated in the RVE.

their molecular weights being: 97, 67, 60, 55, 47, 44, 38, 30, 27, 26 and 20 kD. If the sample is filtered prior to dialysis, the same range of molecular weights is observed, their intensity also being diminished.

As shown in this paper, a protein band of 30 000 daltons persisted throughout all the purification stages, which was also found in varietal apple juices by others researchers (Wall et al., 1996) who discussed the possible relationship of these proteins with haze formation in clarified apple juice (Hsu, Heatherbell, & Yorgey, 1989).

4. Conclusions

A reversed chromatographic method to characterize soluble proteins from cider was proposed. A dialysis procedure followed by liophilization was selected to isolate and concentrate proteins. The present study gives the first indications of the characterization of the proteins found in cider samples.

SDS–PAGE electrophoresis was used to confirm the presence of proteins in the cider samples and to determine their molecular weights. There is one more presence of proteins of low molecular weight and, specifically, a protein band of 30 000 daltons persisted throughout all the purification stages.

It can be inferred that the isolation and characterization of cider proteins may be of great value in the determination of the influence of proteins on foam and haze characteristics.

References

Andrés-Lacueva, C., López-Tamames, E., Lamuela-Raventós, R. M., Buxaderas, S., & De la Torre-Boronat, M. C. (1996). Characteristics of sparkling base wines affecting foam behaviour. *Journal of Agricultural and Food Chemistry*, *44*, 989–995.

Bobe, G., Beitz, D. C., Freeman, A. E., & Lindberg, G. L. (1998). Separation and quantification of bovine milk proteins by reversed-phase high-performance liquid chromatography. *Journal of Agricultural and Food Chemistry*, *46*, 458–463.

Boye, J. I. (1999). Protein-polyphenol interactions in fruit juices. *Recent Res. Devel. Agric. and Food Chem.*, *3*, 85–107.

Brissonnet, F., & Maujean, A. (1993). Characterization of foaming proteins in a champagne base wine. *Am. J. Enol. Vitic.*, *44*, 297–301.

Dorrestein, E., Ferreira, R. B., Laureano, O., & Teixeira, A. R. (1995). Electrophoretic and FPLC analysis of soluble proteins in four Portuguese wines. *Am. J. Enol. Vitic.*, *46*, 235–242.

González-Lara, R., & González, L. M. (1991). Analysis of the soluble proteins in grape musts by reversed-phase HPLC. *Chromatographia*, *32*, 463–465.

González, L. M., & González-Lara, R. (1993). Solid-phase extraction of soluble proteins in grape musts. *Journal of Chromatography A*, *655*, 336–339.

González-Lara, R., & González, L. M. (1996). Hydrophobic interaction-HPLC analysis of soluble grape proteins. *IJBC*, *1*, 309–314.

González-Lara, R., Correa, I., Polo, M. C., Martín-Alvarez, P. J., & Ramos, M. (1989). Classification of variety musts by statistical

analysis of their electrophoretic protein pattern. *Food Chemistry*, *34*, 103–110.

González-Lara, R., Polo, M. C., Correa, I., & Ramos, M. (1989). Características de las proteínas de mostos de uvas de variedades cultivadas en España. *Rev. Agroquím. Tecnol. Aliment.*, *29*, 332–339.

Hancock, W. S., & Sparrow, J. T. (1983). The separation of proteins by reversed-phase high-performance liquid chromatography. *High-Performance Liquid Chromatography*, *3*, 49–85.

Hsu, J. C., Heatherbell, D. A., & Yorgey, B. M. (1989). Effects of fruit storage and processing on clarity, proteins, and stability of Granny Smith apple juice. *Journal of Food Science*, *54*, 660–662.

Knuutinen, J., & Harjula, P. (1998). Identification of fish species by reversed-phase high performance liquid chromatography with photodiode-array detection. *Journal of Chromatography B*, *705*, 11–21.

Laemli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, *227*, 680–685.

Luguera, C., Moreno-Arribas, V., Pueyo, E., Bartolomé, B., & Polo, M. C. (1998). Fractionation and partial characterization of protein fractions present at different stage of the production of sparkling wines. *Food Chemistry*, *63*, 465–471.

Luguera, C., Moreno-Arribas, V., Pueyo, E., & Polo, M. C. (1997). Capillary electrophoretic analysis of wine proteins. Modifications during the manufacture of sparkling wines. *Journal of Agricultural and Food Chemistry*, *45*, 3766–3770.

Pueyo, E., Martín-Alvarez, P. J., & Polo, M. C. (1995). Relationship between foam characteristics and chemical composition in wines and caves (sparkling wines). *Am. J. Enol. Vitic.*, *46*, 518–524.

Polo, M. C., Cáceres, I., Palop, L., Dizy, M., Pueyo, E., & Martín-Alvarez, P. J. (1989). Study of the proteic fraction of grape musts by high performance liquid chromatography and electrophoretical techniques. Variety differentiation. In G. Charalambous (Ed.), *Proceedings of the 6th International Flavor Conference, Rethymnon, Crete, Greece* (pp. 87–101). Amsterdam: Elsevier Science Publishers.

Robillard, B., Despuech, E., Viaux, L., Malvy, J., Vignes-Adler, M., & Duteurtre, B. (1993). Improvements of methods for sparkling base wine foam measurements and effect of wine filtration on foam behaviour. *Am. J. Enol. Vitic.*, *44*, 387–392.

Santoro, M. (1995). Fractionation and characterization of must and wine proteins. *Am.J. Enol. Vitic.*, *46*, 250–255.

Siebert, K. J., Carrasco, A., & Lyunn, P. Y. (1996). Formation of protein-polyphenol haze in beverages. *Journal of Agricultural and Food Chemistry*, *44*, 1997–2005.

Siebert, K. J., Troukhanova, N. V., & Lyunn, P. Y. (1996). Nature of polyphenol-protein interactions. *Journal of Agricultural and Food Chemistry*, *44*, 80–85.

Trujillo, A. J., Casals, I., & Guamis, B. (2000). Analysis of major ovine milk proteins by reversed-phase high performance liquid chromatography and flow injection analysis with electro spray ionisation mass spectrometry. *Journal of Chromatography A*, *870*, 371–380.

Wall, K. M., Tait, V. M., Eastwell, K. C., Reid, C. A., & Beveridge, T. H. J. (1996). Protein in varietally derived apple juices. *Journal of Agricultural and Food Chemistry*, *44*, 3412–3415.

Waters, E. J., Wallage, W., & Williams, P. J. (1991). Heat haze characteristics of fractionated wine proteins. *Am. J. Enol. Vitic.*, *42*, 123–127.

Yokotsuka, K., Nozaky, K., & Takayanagi, T. (1994). Characterization of soluble glicoproteins in red wine. *Am. J. Enol. Vitic.*, *45*, 410–416.