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Technical report

A novel preparation technique of red (sparkling) wine for protein analysis

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ABSTRACT

Despite their low concentration, proteins can influence several key enological parameters such as foam stability or haze formation in (sparkling) wine. Most studies focus on white (sparkling) wine since the higher content of phenolic compounds in red wines impairs proteomic research. The aim of the study was the development of a method for the preparation of red (sparkling) wine proteins for proteomic analysis. Three methods of sample preparation were assessed on silver stained SDS-PAGE gels and with MALDI-TOF MS. Our new method was highly suitable for the preparation of proteins for the aforementioned applications. The results showed a substantial increase in signal intensity with a simultaneous decrease in background noise. The preparation protocol consists of (i) dialysis and freeze drying of the sample, (ii) removal of phenolic compounds by water-saturated phenol and (iii) protein precipitation by addition of ammonium acetate. Employment of this method followed by SDS-PAGE analysis allowed for silver stained gels with diminished background or streaking and clearly resolved protein bands. Analysis of spectra obtained from samples prepared according to the proposed protocol showed increased intensity and signal-to-noise ratio in MALDI-TOF MS. Furthermore it was demonstrated that this method can be applied to various kinds of grape products.

1. Introduction

Although proteins are not the major components in (sparkling) wines, they contribute essentially to its quality. Several previous studies focussed on the role of these macromolecules. They influence the foaming properties of sparkling wines [1,2], interact with wine aroma compounds [3,4], affect the tartaric stabilization [5,6] or influence the formation of haze in white wines [7–9]. This shows the necessity of profound analysis of the protein composition present in (sparkling) wines with the goal of quality improvement. Due to the low protein content in (sparkling) wine [10] and the abundance of phenolic substances especially in red wine [11] protein analysis of red (sparkling) wine is impaired. As reviewed by Moreno-Arribas et al. [12] several methods have been established for the preparation and characterization of wine proteins including dialysis, ultrafiltration, precipitation, SDS-PAGE, IEF, 2D or capillary electrophoresis, size exclusion chromatography, affinity or reversed phase chromatography or FPLC. However, almost every study concerning wine protein analysis has been conducted with white (sparkling) wines. This fact can be attributed to limitations in analytical methods regarding the high content of phenolic compounds in red wines. In order to properly examine the protein composition of red (sparkling) wines, an alternative approach for the preparation of wine proteins and the removal of interfering compounds is needed. Therefore the purpose of this study was to develop, optimize and establish an effective method for red (sparkling) wine protein preparation, which can be used in the assessment of proteins on silver stained SDS-PAGE gels as well as by MALDI-TOF MS analysis.

We employed three different techniques for the preparation of proteins from red wine samples. In method A, 50 ml of (sparkling) wine were dialyzed against 20 times the volume of deionized water in dialysis tubes (MEMBRA-CEL®, MWCO 3500, Serva Electrophoresis GmbH) for 72 h in order to remove low molecular compounds such as glycerin, ethanol and residual sugars. The retentates were lyophilized and the resulting lyophilizates stored at −20 °C until further use. In method B, samples were treated according to method A followed by resuspension in extraction buffer (0.1 M Tris-HCl (pH 8.8), 10 mM EDTA, 0.4% (v/v) β-mercaptoethanol, 10% (w/v) DTT, 100 mM KCl) and protein precipitation with three times the volume of 0.1 M ammonium acetate in

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methanol over night at −20 °C. Protein pellets were separated from the supernatant (centrifugation: 3000*g*, 10 min, 4 °C) and washed with 80% acetone. Subsequently the supernatant was discarded (centrifugation: 3000*g*, 10 min, 4 °C) and the resulting pellet was air-dried and stored at −20 °C. Method C was based on the method of Hurkman et al. [13] for the extraction of interfering phenolic compounds. Method B was extended by the addition of the same volume of water-saturated phenol to the extraction buffer followed by 30 min of shaking at 4 °C and a subsequent phase separation by centrifugation (6000*g*, 15 min, 4 °C). Furthermore, hexane, butanol, and phenol/chloroform/isoamyl alcohol (25:24:1) were tested as organic solvents. The lower phenolic phase was recovered and washed once with extraction buffer. Moreover, the effect was tested of the omission of washing as well as the integration of up to four successive washing steps. Protein precipitation with ammonium acetate was achieved as described in method B. After centrifugation (20,000*g*, 40 min, 4 °C) the protein pellet was successively washed with 0.1 M ammonium acetate with 10 mM DTT in methanol and 10 mM DTT in 80% ice cold acetone (both times incubation: 60 min, 4 °C; centrifugation: 13,000*g*, 30 min, 4 °C). The supernatant was discarded and the protein pellet air-dried and stored at −20 °C. For further analyisis, the untreated lyophilizates (method A) and the protein pellets obtained with methods B and C were resuspended in Laemmli application buffer (0.25 M Tris-HCl (pH 8.46), 7.5% (w/v) SDS, 25% (v/v) glycerine, 0.25 mg/ml bromophenol blue, 12.5% (v/v) β −mercaptoethanol) for SDS-PAGE or in organic solvent (50% ACN, 2.5% TFA) for MALDI-TOF MS analysis.

SDS-PAGE is a well know method for the analysis of proteins. Nevertheless this technique is susceptible for interfering substances such as polyphenols present in red (sparkling) wines. To the best of our knowledge, only two previous publications have addressed the analysis of the protein composition in red wine by SDS-PAGE [14,15]. These authors used PVP(P) to decrease the concentration of phenolic compounds in their samples. However, in our experiments these substances also reduced the protein content of samples significantly. As a consequence we tested other established wine preparation methods such as dialysis and lyophilization [16–18] or salting-out precipitations [19,20] combined with the application of organic solvents. Assessment of these methods using silver stained SDS-PAGE gels revealed a high background and a low resolution of protein bands (Fig. 1). Vertical SDS-PAGE (separating gel = 16% T, stacking gel = 4% T) was performed in a Mini-PROTEAN® Tetra Cell Electrophoresis System (Bio-Rad Laboratories GmbH, München, Germany) according to the method of Schägger and von Jagow [21]. The electrophoresis was conducted under a constant voltage of 100 V for 120 min at room temperature. A molecular marker (Spectra Multicolor Low Range Protein Ladder; Thermo Fisher Scientific Inc., St Leon-Rot, Germany) was loaded simultaneously with the samples in each run. Prior to their application onto the gel, samples were diluted in Laemmli buffer to a final concentration of 3% of the original aliquot. For the application onto the gels 10 μl of each sample were used. After the electrophoretic run, gels were silver stained according to the method of Blum et al. [22]. Samples prepared by dialysis and lyophilization showed an extreme background with the result that no separated protein bands could be detected (Fig. 1A). A combination of this method with subsequent protein precipitation reduced the background slightly so that some protein bands became visible but a clear distinction was not achieved (Fig. 1B). Hence a novel preparation technique of red wine for protein analysis was optimized and established. The protocol was composed of dialysis and lyophilization followed by a phenol extraction of proteins with water-saturated phenol and precipitation of proteins by addition of ammonium acetate in methanol. During the optimization of this protocol several numbers of washing steps as well as various organic solvents were tested. Best results were obtained by using one washing step and the use of water-saturated phenol as organic solvent. Silver stained SDS-PAGE gels of samples prepared by the proposed method showed a minimum of background and streaking on the gels as well as clearly resolved bands (Fig. 1C). Several runs were conducted to demonstrate the reproducibility of the novel method. Although the preparation is time consuming, the improvement of preparation of red wine proteins is convincing. Following the establishment of the new protocol for red sparkling wines, other grape-based beverages (Table 1) were analyzed. Although the polyphenol content of white and rosé wine is low as compared to red wine [23], SDS-PAGE analysis can still be impaired by it. We demonstrated that the novel preparation technique is also suited for all kinds of wines as well as grape juices. In the right section of Fig. 1 a compilation of selected lanes from different SDS-PAGEs is displayed (for entire gels see supplementary material). Samples treated with this method prior to SDS-PAGE analysis showed clear protein bands and a reduced background in silver stained gels. Thus, the method can be applied for the proteomic analysis of all kind of wines. It may also enable a comparison of the protein composition of different wine cultivars or of wines obtained by different production processes using SDS-PAGE analysis. In further studies, MALDI-TOF MS was assessed as a powerful technique for the characterization of several biomolecules in wine. Literature shows that, similar to SDS-PAGE this powerful analytical tool has been nearly exclusively been applied to the analysis of white wines [24,25]. Again, this may be attributed to the interfering effects of polyphenols. To the best of our knowledge only Carpentieri et al. [26] and Nunes-Miranda et al. [27] have so far been the only authors to perform direct MALDI-TOF MS experiments with red wine samples. Nevertheless the authors did not attempt to analyse the protein composition of red wines but were aiming at pigments or volatile compounds. We performed MALDI-TOF MS analysis of red wine proteins prepared

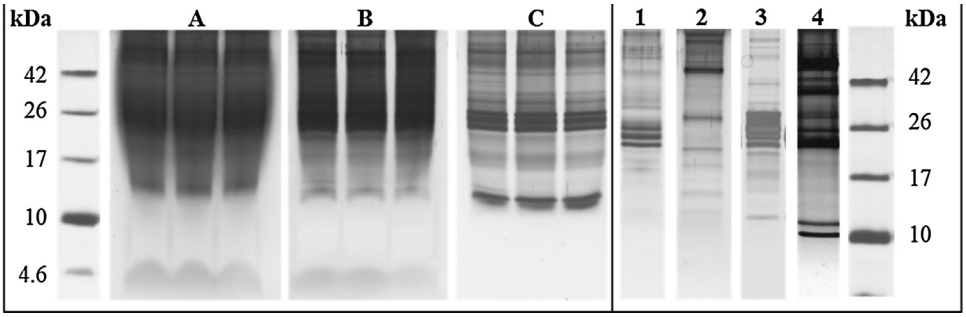

Fig. 1. SDS-PAGE of red sparkling wine obtained with three different preparation techniques for the same sample. Proteins are visualized by silver staining. (A) Method A: Dialysis and lyophilization of the sparkling wine. (B) Method B: Protein precipitation with ammonium acetate after method A. (C) Method C: Combination of method B with extraction of phenols by use of water-saturated phenol. Application of method C for (1) white wine, (2) red wine (3), Rosé wine Weißherbst and (4) red grape juice.