Technical report

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

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1. Introduction

Although proteins are not the major components in (sparkling) wines, they contribute essentially to its quality. Several previous studies focused 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 impaire. 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 glycercin, ethanol and residual sugars. The retentates were lyophilized and the resulting lyophilizates stored at –20 ºC 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: 3000g, 10 min, 4 °C) and washed with 80% acetone. Subsequently the supernatant was discarded (centrifugation: 3000g, 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 (6000g, 15 min, 4 °C). Furthermore, hexane, butanol, and phenol/chloroform/isooamy 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 (20,000g, 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: 13,000g, 30 min, 4 °C). The supernatant was discarded and the protein pellet air-dried and stored at –20 °C. For further analysis, 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 Weißerherbst and (4) red grape juice.](image-url)
according to the three different preparation techniques previously tested with SDS-PAGE analysis. In contrast to the studies about red wine mentioned above we used sinapinic acid (SA) as a matrix instead of 2,5-dihydroxybenzoic acid (DHBA) or α-cyano-4-hydroxy-cinnamic acid (HCCA). A comparison of these three matrices revealed the highest intensity in the mass to charge ratio when SA matrix was used. The matrix for MALDI-TOF MS analysis was prepared by dissolving 10 mg of SA in 1 ml organic solvent (50% ACN, 2.5% TFA). For measurements, 1 µl of matrix was applied onto the target and successively coated after air-drying with 1 µl of sample and addition of another 1 µl of matrix successively. Analyses were performed using a microflex LT MALDI-TOF mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany) equipped with a nitrogen laser (λ = 337 nm) operating in linear positive ion detection mode using the MALDI Biotyper 2.0 software (Bruker Daltonics GmbH, Bremen, Germany). Mass spectra (2000–20000 Da) were acquired manually at each spot position by accumulating 240 laser shots per sample. Processing of spectra was done with the FlexAnalysis 3.3 software package (Bruker Daltonics GmbH, Bremen, Germany). Suitability of each protein preparation method was determined after evaluation of signal-to-noise-ratio and signal intensity. Spectra obtained after applying methods A and B contain almost no peaks and the signal-to-noise ratio was too low for meaningful analysis of results (see Fig. 2A and Fig. 2B). With the newly developed technique (method C) non-protein contaminants, especially polyphenols, were excluded from extracts resulting in spectra with a higher intensity and signal-to-noise ratio as compared to methods A and B (Fig. 2C). To rule out that this is not an experimental artefact, the experiment was repeated three times. Results showed that spectra obtained after applying method A or B always display almost no peaks, whereas spectra obtained after applying method C display several peaks with higher intensity. We assume an interfering influence of

| Table 1: Selected wines for protein preparation and subsequent SDS-PAGE or MALDI-TOF MS analysis. |
|---------------------------------------------------------------|
| **Type of wine**       | **Grape variety** | **Vintage** | **Origin**           |
| Red sparkling wine    | Cuvee            | –           | –                    |
| White wine            | Riesling         | 2014        | Neustadt, Germany    |
| Red wine              | Spätburgunder    | 2013        | Geisenheim, Germany  |
| Rosé wine, Weißherbst | Lemberger        | 2012        | Badenwürttemberg, Germany |
| Carbonated grape juice| Dornfelder       | –           | Badenwürttemberg, Germany |

Fig. 2. MALDI-TOF MS spectra of red sparkling wine obtained with three different preparation techniques for the same sample. (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.
polyphenols resulting in quenching of the protein peaks in MALDI-TOF MS analysis.

It could be shown that the developed preparation method for wine proteins is an effective tool for the removal of non-protein contaminants from samples. Application of this method before SDS-PAGE analysis results in more distinguishable protein bands and a reduction in background noise. In MALDI-TOF MS analysis the proposed procedure strongly improved signal-to-noise ratios and increased signal intensities. The developed method enables a comparison of different beverages made of grapes in regard to their protein composition. The new protocol development during our study provides an important new tool for protein preparation which allows for highly resolved proteome analysis of red (sparkling) wines and grape products. Moreover, this preparation method may also be considered for other proteomic workflows such as HPLC.

2. Conflict of interest

On behalf of all authors of this manuscript the corresponding author declares that there is no conflict of interest of any kind regarding the publication of our manuscript entitled “A novel preparation technique of red (sparkling) wine for protein analysis” in EuPA Open.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.euprot.2016.03.001.

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