TOSOH

Application Note

HPLC Analysis of lysozyme in different types of wine

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Hen's egg white lysozyme is used in winemaking to eliminate lactic acid bacteria and to control malolactic fermentation. As eggs and derivatives are regarded as potential allergenics the European Commission issued a directive that wine labels must include information about egg derived ingredients like lysozyme. As a consequence the International Organisation for Wine and Vine (OIV) published a standard method for measurement of lysozyme in wine by HPLC, which is based on a method developed at the University of Bologna.

'A bottle of wine contains more philosophy than all the books in the world'. It was Louis Pasteur (1822-95) the famous French chemist and microbiologist, who stated this. Wine was one of his early objects of study and he solved the mystery of fermentation when he identified and isolated the specific micro organisms responsible for normal and abnormal fermentations in winemaking. His work remains to be the basis of today's winemaking technology.

THE ROLE OF EGG LYSOZYME IN WINEMAKING

Lysozyme (E.C. 3.2.1.17) isolated from egg whites has a long tradition as an antimicrobial agent used in food industry1. It is an enzyme with muramidase activity which degrades the cell wall of gram-positive bacteria such as Oenococcus, Pediococcus, and Lactobacillus. In the past it was used mainly as preservative in cheese making, to prevent spoilage by micro organisms. Although micro organisms are mostly regarded as food spoilage some are essential for fermentation processes. During winemaking the primary fermentation process converts the grape sugar to alcohol by yeast. The so called malolactic fermentation occurs shortly after the end of the primary fermentation. Lactic acid bacteria convert L-malic acid, to, L-lactic acid in such a way acting as biological deacidifiers. In red wines, malolactic fermentation could hence results in a more balanced wine, while for white wines, where the acidic notes should be preserved, its intervention is often to be avoided. A tight control of this process is however necessary because the onset of malolactic fermentation in the bottle is undesirable as the process could proceed to further metabolize other acids (citric and tartaric acids), thus increasing acetic acid amounts. Furthermore the wine will appear to the consumer to still be fermenting and the wine may also lose its flavour integrity and take on an unpleasant lactic aroma.

CONTROL OF MALOLACTIC FERMENTATION

In the past cooling and the anti-microbial agent sulphur dioxide were used to inhibit the growth of lactic acid bacteria. Sulphur dioxide can provoke allergic reactions like headache in susceptible individuals. In Europe wines which contain more than 10 ppm of sulphur have to be labelled accordingly. As lysozyme can lyse the cell wall of wine lactic acid bacteria, it provides a practical means for delaying or preventing the growth of Oenococcus oeni and consequently the onset of malolactic fermentation[2]. To control the malolactic fermentation during vinification and subsequent bottling the addition of up to 500 mg lysozyme per liter is permitted since 2001 by OIV. Lysozyme cannot replace sulphur dioxide completely because it is lacking the anti-oxidative effect of sulphur dioxide. It can, however greatly reduce the amount of sulphur dioxide needed to achieve microbial stability over the life of both red and white wines.

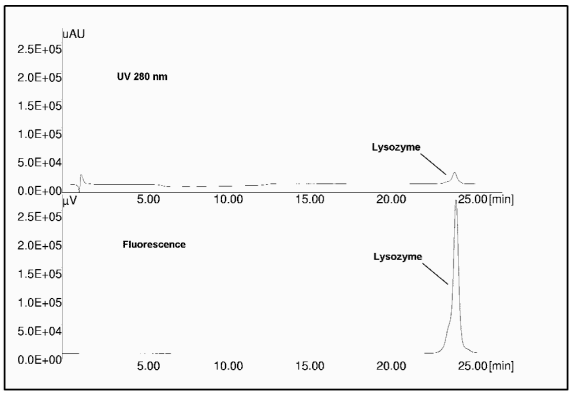

Figure 1

Chromatogram of a model solution (water and tartaric acid 2g/l) spiked with pure lysozyme (final concentration 150 mg/l). A: UV @ 280nm; B: Fluorescence @ 276ex/345em

Detection	Linearity range (mg/L)	LOD (mg/L)	LOQ (mg/L)	Repeatability (white wine, N = 5) RSD %	Repeatability (red wine, N = 5) RSD %
UV 280	5 - 250	1.86	6.20	0.62	5.54
FLD	1 - 250	0.18	0.59	0.68	2.37

Table 2

LABELLING OF POTENTIAL ALLERGENIC SUBSTANCES IN EUROPE

Today, eggs and their derivatives are regarded as potential allergenics. In the first European directive about the labelling of allergenic ingredients in food (Directive 2000/13/EC), lysozyme used in winemaking was provisionally excluded. In 2007 the European Commission issued the directive 2007/68/EC including the final list of ingredients which must be indicated on the label of food stuffs including alcoholic beverages as they are likely to cause adverse reactions in susceptible individuals. Now lysozyme is part of the list and therefore needs to be determined in the final product. All wines which will be labelled and marketed after 31 May 2009 have to be labelled accordingly. As a consequence the International Organisation for Wine and Vine (OIV) published a standard method[3] for measurement of lysozyme in wine, based on a HPLC method with fluorometric detection, developed at the Food Science Department of the University of Bologna[4].

DETERMINATION OF LYSOZYME BY HPLC

Early methods for the determination of lysozyme used microbiological tests based on enzyme activity. But as these methods were not very robust and very sensitive to phenolic or colloid interactions, alternative HPLC methods were developed. HPLC-UV methods[5-7] did not reach the sensitivity required to detect residual lysozyme in marketed wines in the low concentrations, possibly sufficient to cause allergenic responses in sensitive consumers. The new validated HPLC-FLD method is based on the separation of the components on a polymer based TSK-GEL Phenyl-5PW reversed phase column with 1000 Å pore size, which is especially suited for the separation of proteins. Fluorometric detection of the lysozyme resulted in an increased sensitivity compared to UV based HPLC methods. It allows the quantification of lysozyme independently of the enzyme activity.
Fluorometric detection (FLD) of the enzyme without derivatisation is based on the intrinsic fluorescence of its phenylalanine, tyrosine and tryptophan residues. It is dominated by tryptophan because of its higher molar extinction[8]. Egg white lysozyme has six tryptophan residues, resulting in a much higher fluorescence signal intensity compared to the common UV detection at 280 nm. For spectrofluorometric detection an excitation wavelength of 276 nm and an emission wavelength of 345 nm were applied.

HPLC ANALYSIS OF WHITE, ROSE AND RED WINE AFTER THE ADDITION OF LYSOZYME

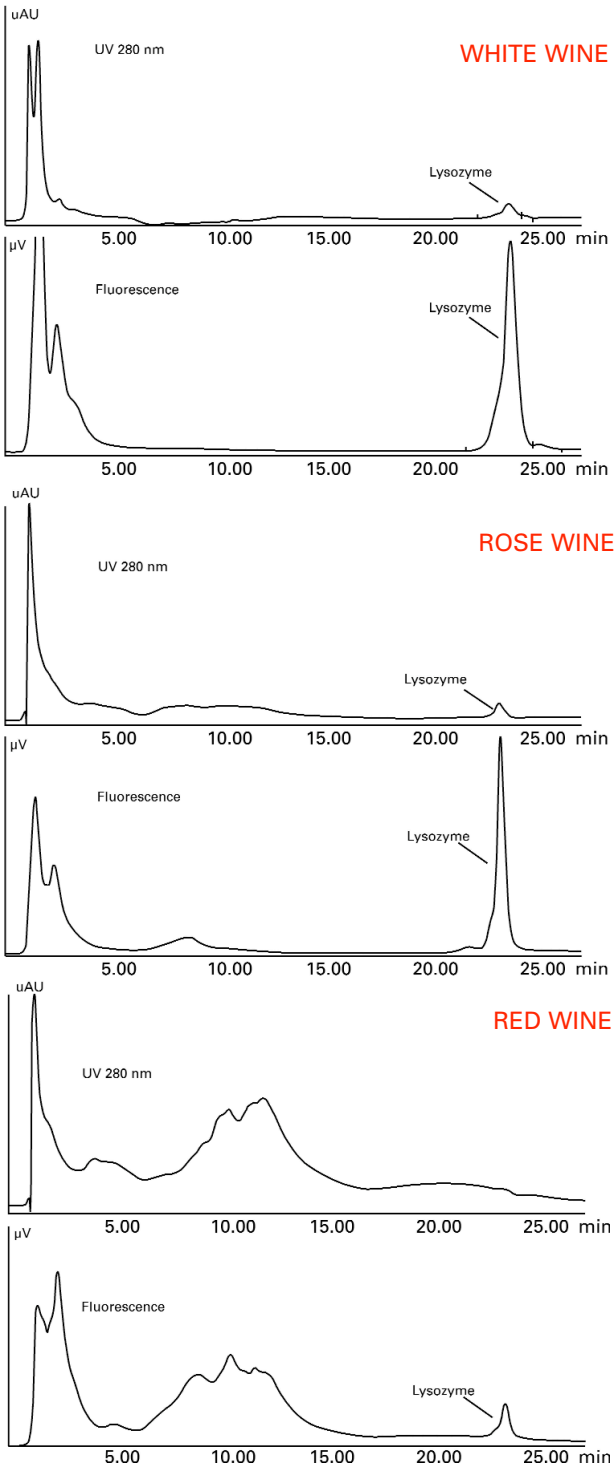

Figure 2

HPLC analysis of three Italian wines after the addition of lysozyme: A: white, B: rosé and C: red

Limits of detection (LOD) and of quantification (LOQ) are up to 11-fold lower compared to UV at 280 nm (Table1). Figure 1 shows the UV280 and fluorescence chromatograms of a model solution spiked with lysozyme to a final concentration of 150 mg/l.

In the original work[4] it was found that a matter of concern in analysis of lysozyme in wines is the portion of enzyme bound by phenols or other compounds. This happens especially in red wines. Fluctuations related to these interactions can be reduced by acidification of the sample. Sample pretreatment with HCL enhanced the recovery of lysozyme in red wine. The highest recovery was reached with 10 N HCL at the ratio of 1:10 (HCL : wine). To reach the recoveries reported in the original paper, HPLC injection immediately followed acidification and filtration. Nevertheless repeatability of the method for red wines is worse than for white wines, due to higher concentrations of interfering substances.

We analysed white, red and rosé Italian wine to show the versatility of this method.

MATERIAL & METHODS

HPLC system:	Binary pump, 20 µl loop, column oven, photodiode array detector, fluorescence detector (Jasco,Tokyo, Japan)
Column:	TSKgel Phenyl-5PW RP reversed phase column, 4.6 mm ID x 7.5 cm L (Tosoh Bioscience, Stuttgart, Germany)
Eluents:	A: 98.8 % Water/1% Acetonitrile/ 0.2% TFA; B: 70% Acetonitrile/29.8% Water/ 0.2%TFA
Gradient:	100% A for 3 min, to 35% B in 7 min, maintained for 5 min, to 59.5 % B 0in 12 min, to 100% B in 2 min, maintained for 5 min, to 100% A in 2 min, maintained for 10 min.
Flow rate:	1 ml/min
Temperature:	30°C
Detection:	UV @ 280nm, Fluorescence @ $276_{ex}/345_{em}$ Gain=10
Injection vol.:	20 µl
Sample:	Trebbiano di Romagna white wine, Montepulciano di Abruzzo rosé wine, Nero d'Avola red wine, each spiked 24 hours prior to analysis with lyzozyme to a final concentration of 160 mg/l.
Sample pre-treatment:	Samples were acidified (10:1) with HCL (10 M), filtrated after 5 min. using a 0.22 µm disposable filter and injected directly after filtration.

RESULTS

We analysed three different types of wine from Italy to which lysozyme had been added in the laboratory, with the aim to testing the method. The white wine was a Trebbiano di Romagna, the rosé a Montepulciano di Abruzzo and the red one a Nero d'Avola. The chromatograms are shown in Figure 2. Red wine strongly binded the protein (probably due to the higher presence of tannins and pectins) and a very low amount of free lysozyme was found after 24 hours from the addition. However, free lysozyme tends to progressively increase with time and the final amount of free lysozyme in red wines is usually about 40 - 60% of the added quantity. The asymmetry (fronting) observed in the chromatograms of the samples might be related to the portion of lysozyme which has reacted with other components, such as tannins, sulphur dioxide or pectins. The exact nature of this portion is still unknown and will be the subject of next studies. The peak shape progressively degrades while the lysozyme is interacting with the matrix.

WINE	LYSOZYME (mg/l)
White wine	147
Rose wine	135
Red wine	6.5

Table 2

Results of the HPLC-FLD analysis of three different types of wine spiked with lysozyme (final amount: 160 mg/L)

HPLC-FLD analysis of underivatised hen's egg lysozyme in wines can be applied to all types of wine. It shows a high sensitivity and is a straight forward method for quantification of free amounts of enzyme during winemaking.

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