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Ergosterol as an objective indicator for grape rot and fungal biomass in grapes

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ABSTRACT

Sound grapes are an essential prerequisite for the production of high-quality wines. However, pricing of grapes is so far mainly based on the must weight of grape deliveries, although e.g. highly botrytised grapes become raisined bringing about higher contents of soluble solids than sound ones. Besides the desired 'noble rot', in particular infection of unripe fruits by grape rot decreases the perceptual quality by destroying fruit flavours typical of the grape variety, furthermore leading to off-flavours, off-odour, bitterness and colour-loss. Moreover, the formation of mycotoxins, in particular ochratoxin A, associated with fungal infestation highly affects food safety of the products. Consequently, there is a strong need of an objective assessment of the phytosanitary status of grapes to be used for vinification and other grape derived products. To date, the commonly applied visual examination is subjective, and particularly challenging when grapes were mechanically harvested. Objective methods suggested in the literature, such as the analysis of major and secondary metabolites of moulds and the determination of related enzyme activities, are requiring tedious sample preparation being generally time-consuming. Furthermore, these methods are prone to interferences, and mostly lacking selectivity and sensitivity.

Ergosterol is a characteristic component of the fungal cell membrane, while it is completely or nearly absent in animal, plant and bacterial cells. Thus, our group proposes ergosterol as a specific and quantitative marker for fungal infection of grapes. Mould strains relevant for grape rot were demonstrated to contain considerable amounts of ergosterol. In grapes infected with several pure mould strains and in naturally contaminated grapes, ergosterol was unambiguously detected and quantitated. In contrast, only traces were found in sound grapes, presumably originating from the presence of ubiquitous yeasts and moulds. Ergosterol levels highly correlated with the degree of fungal decay in blended samples of mashes made from sound and rotten berries. An analytical HPLC-method for the quantitation of ergosterol was developed allowing a simple and objective evaluation of the phytosanitary status of grapes, thus enabling fair and incentive payments between grape growers and wineries.

1. Introduction

In general, vinification of sound grapes is an essential requirement for the production of high-quality wines with sweet wines, where botrytised grapes ('noble rot') are desired, being the sole exception. In contrast, grape rot commonly decreases the perceptual quality of wine made from rotten grapes. *Botrytis cinerea*, *Penicillium* spp. and other rots destroy fruit flavours typical of the grape variety (Dittrich, 1989), and often lead to musty, earthy or mouldy off-flavours and bitter taste (Ribéreau-Gayon, Dubourdieu, Donèche, & Lonvaud, 2006; Scott, Dambergs, & Stummer, 2010; Walter, 2012). Combinations of various filamentous fungi, oxidative yeasts, and acetogenic bacteria cause sour rot making infected grapes unsuitable for wine production (Ribéreau-Gayon et al., 2006; Scott et al., 2010). Infections of grapes with *B. cinerea* or powdery mildew (*Erysiphe necator* Schw., syn. *Uncinula necator* (Schw.) Burr.) result in notable browning of white and red varieties and substantial colour-loss in red varieties (Dittrich, 1989; Scott et al., 2010).

Besides alterations of the perceptual quality attributes, chemical composition of grapes changes due to the degradation of sugars and acids and the production of microbial metabolites such as glycerol and gluconic acid (Ribéreau-Gayon et al., 2006). Moreover, the microbial formation of biogenic amines and mycotoxins is a

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major food safety concern due to their toxicity affecting human health. Biogenic amines like the histamine have high biological activity in humans, causing adverse effects (Flamini & Larcher, 2008). Increased content of biogenic amines was reported in grapes infected with *B. cinerea* and *Penicillium* spp. and the derived must and wine (Kiss, Korbász, & Sass-Kiss, 2006). Furthermore, *Penicillium expansum* and other grape-infecting *Penicillium* species may produce patulin (Abrunhosa, Paterson, Kozakiewicz, Lima, & Venancio, 2001) causing severe toxicosis with gastrointestinal disorders and other toxic effects (Barkai-Golan, 2008b). After the addition of sulphur dioxide and fermentation of must, patulin was no more detectable (Altmayer, Eichhorn, & Plapp, 1982; Scott, Fuleki, & Harwig, 1977). Additionally, technological problems during vinification e.g. impaired fermentation and hindered clarification, may result from grape rot (Ribéreau-Gayon et al., 2006).

Being possibly human carcinogenic, ochratoxin A (OTA) represents one of the most potent mycotoxins (Barkai-Golan, 2008a). Recent studies indicate that Aspergilli belonging to section *Nigri* are the main OTA producers in grapes. In particular, *Aspergillus carbonarius* is considered as the major source of OTA in grapes with the putative contribution of *Aspergillus tubingensis*, and *Aspergillus niger* (Barkai-Golan, 2008a; Battilani & Silva, 2010).

As described above, infected grapes are highly undesirable for wine production. Since the commonly used visual assessment of fungal contaminations is subjective and impractical, particularly for mechanically harvested grapes, there is a strong need for a reliable, objective assessment of the phytosanitary status of grapes suitable for a profound quality examination at the early stage of raw material delivery. For this purpose, metabolites such as ethanol, ethyl acetate, glycerol, acetic, citric, gluconic and galacturonic acids, laccase activity, and secondary metabolites such as OTA have been suggested as indicators of fungal infestation (Dewey, Hill, & DeScenzo, 2008; Grassin & Dubourdieu, 1989; Zoecklein & Gump, 2010).

Further biochemical methods for assessing fungal contamination were based on pathogen-specific DNA detection, namely DNA hybridisation and polymerase chain reaction (PCR) assays as well as enzyme-linked immunosorbent assays (ELISA) (Scott et al., 2010). Microbiological inspections using light microscopy yielding the Howard Mould Count (HMC) are also used, although being of low precision (Grasselli, Leoni, Sandei, & Mori, 1993; Kadakal, Tağı, & Artık, 2004). Plate count techniques are time-consuming and restricted to the detection of living fungi (Gourama & Bullerman, 1995b). Determination of both chitin and adenosine triphosphate (ATP), being more or less specific constituents of moulds, may be used to estimate fungal growth due to their abundance in fungi. However, due to the co-occurrence of chitin and ATP in insects and all living cells, respectively, both methods suffer from comparatively low sensitivity and reproducibility (Gourama & Bullerman, 1995b).

Exhibiting much higher specificity, ergosterol (ergosta-5,7,22-trien-3β-ol) occurring in the vast majority of fungi within the Asco- and Basidiomycota is a characteristic component of their cell membrane, while plant, animal, and bacterial cells are completely or virtually devoid of ergosterol (Weete, Abril, Blackwell, & Butler, 2010; Weete & Gandhi, 1996). Therefore, ergosterol was previously shown to be a suitable marker for estimating fungal biomass and mould contamination in various plant materials (Gessner, Bauchrowitz, & Escautier, 1991; Gutarowska & Żakowska, 2010; Richardson & Logendra, 1997), atmospheric aerosols (Lau, Lee, Chan, & Fang, 2006), grass seeds (Richardson & Logendra, 1997), and soils (Montgomery, Monreal, Young, & Seifert, 2000; Ruzicka, Edgerton, Norman, & Hill, 2000; Stahl & Parkin, 1996). Particularly, ergosterol has been suggested for the determination of fungal contamination in tomato products (Battilani, Chiusa, Cervi, Trevisan, & Ghebbioni, 1996; Kadakal et al., 2004; Sio et al., 2000), and apple juice (Kadakal, Nas, & Ekıncı, 2005). To the best of our knowledge, reports on the use of ergosterol as a phytosanitary marker of grape contamination are so far missing. Additionally, ergosterol has often been suggested as a readily measurable indicator of mycotoxins in agricultural commodities like grains (Gourama & Bullerman, 1995a; Olsson, Börjesson, Lundstedt, & Schnürer, 2002; Pietri, Bertuzzi, Pallaroni, & Piva, 2004; Saxena, Munimbazi, & Bullerman, 2001), dried figs (Karaca & Nas, 2006), and apple juice (Kadakal et al., 2005).

Hence, the aim of our study was to establish and validate ergosterol as an objective and reliable fungal marker to estimate the degree of spoilage and the amount of fungal biomass in grape mashes. Pure mould strains relevant for grape rot, grapes infected under defined conditions by inoculation with the same mould strains, and a large selection of grapes naturally contaminated on the vineyard should be analysed for their ergosterol content. Additionally, further specific ingredients should be examined. Consequently, a quantitative indicator of grape rot allowing the reliable quality assessment of grapes and fair pricing of the delivered raw material for juices and fermented products intended for human consumption should be established.

2. Material and methods

2.1. Sample material

2.1.1. Mould sample material

Ten different strains of 5 mould species relevant for grape rot were analysed for their ergosterol contents (Table 1). They were obtained from the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands (CBS), the Dienstleistungszentrum Ländlicher Raum Rheinpfalz, Neustadt an der Weinstraße, Germany (DLR), and the Leibniz Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures), Braunschweig, Germany (DSM). Moulds were cultivated in 2% malt extract broth at 21 °C for 4 days; the mycelia were harvested and freeze-dried. The dried samples were homogenized with a mortar and pestle, and stored in a desiccator until analysis.

2.1.2. Infection of grapes with pure strains of moulds

Riesling grapes from two vintages were obtained from two different cultivation areas (Stuttgart-Hohenheim, Germany, in 2011; and Neustadt an der Weinstraße, Germany, in 2012). Bunches of grapes were split, superficially sanitised by subsequent immersion into 70% ethanol, 0.35% sodium hypochlorite, and 70% ethanol for 30 s, 2 min and 30 s, followed by air-drying as described by Coertze and Holz (1999). Berries were scarified with an injection

Table 1
Ergosterol contents (w_{Erg}) in fungal dry mass and biomass conversion factor (BCF) of different mould species relevant for grape rot.

Sample	Species	Isolate[a]	w_{Erg} [mg/g][b]	BCF [g/mg]
AC1	*Aspergillus carbonarius*	CBS 111.26	2.32 ± 0.00	0.43
AN1	*Aspergillus niger*	CBS 119.558	2.44 ± 0.05	0.41
AN2	*Aspergillus niger*	DLR 8562	2.30 ± 0.05	0.44
BC1	*Botrytis cinerea*	DLR 9523/1	1.71 ± 0.00	0.59
BC2	*Botrytis cinerea*	DLR 8850/2	1.98 ± 0.00	0.50
PE1	*Penicillium expansum*	DLR 9732	3.49 ± 0.06	0.29
PE2	*Penicillium expansum*	DSM 62841	3.47 ± 0.02	0.29
PE3	*Penicillium expansum*	DLR 10/1	3.56 ± 0.05	0.28
TR1	*Trichothecium roseum*	DLR 9727	2.85 ± 0.05	0.35
TR2	*Trichothecium roseum*	DLR 9724	2.47 ± 0.00	0.40

[a] See Section 2.1.1.
[b] Values are means ± standard deviation.