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Solid phase extraction, multidimensional gas chromatography mass spectrometry determination of four novel aroma powerful ethyl esters Assessment of their occurrence and importance in wine and other alcoholic beverages

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Abstract

A method for the quantitative determination of four powerful aromatic ethyl esters recently identified in some wines has been developed, validated and applied to the determination of these compounds in different samples of wine, whisky and brandy. Ethyl 2-, 3-, and 4-methylpentanoate and ethyl cyclohexanoate are extracted from 100 ml of sample by solid phase extraction (SPE) on a 200 mg LiChrolut EN bed. Major compounds are eliminated by rinsing with a water–methanol (50:50) solution containing 1% sodium bicarbonate, and analytes are eluted with 1.5 ml of dichloromethane. Fifty microlitres of this extract are then injected in a multidimensional gas chromatography–mass spectromety (GC–GC–MS) system. Recoveries in the SPE are quantitative. Method repeatability is satisfactory (5–12% for a 5–10 ng l^{-1} level, and less than 7% for 25–50 ng l^{-1} level), the method linearity holds along the whole range of occurrence of analytes (2–2700 ng l^{-1}), and the signal is independent on the matrix. Method detection limits are below 1 ng l^{-1} in all cases. Results suggest that these compounds are formed by the slow esterification with ethanol of the corresponding acids formed by different microorganisms. The levels of these compounds are above the corresponding thresholds in most samples of aged wines or distillates, but are particularly high in some sweet wines, whiskeys and brandies where they may constitute the most important contributors to the sweet-fruity notes reaching concentrations up to 85–350 times higher than the corresponding odor thresholds.

Keywords: Wine; Distilled beverages; Whisky; Brandy; Cognac; Aroma; Flavour; Ethyl 2-, 3- and 4-methylpentanoate; Ethyl cyclohexanoate; SPE; GC–GC; GC–MS

1. Introduction

Esters are abundant volatile constituents of different foods and beverages such as fruits and fruit juices, olive oil, fermented dairy derivates, beer, wine or distilled alcoholic beverages [1–6]. During alcoholic fermentation, a great number of esters can be generated as a result of yeast metabolism, but the most abundant are essentially ethyl esters of organic acids, acetates of fusel alcohols and ethyl esters of fatty acids [7]. The ethyl ester content of alcoholic beverages increases during aging, as a consequence of the slow esterification of different organic acids with ethanol. Some of the ethyl esters can be found at concentrations above their odor threshold and their aroma role is well documented in the scientific literature. This is particularly true for the major ethyl esters of fatty acids [5,7], although some other minor ethyl esters, such as the ethyl esters of 2-methyl and 3-methylbutyric acids may also play some role [8–11].

Recent research by gas chromatography-olfactometry has given first evidence that some aged fortified wines contain other novel and naturally rare ethyl esters which may have some impact on their aroma [12,13]. Such compounds are ethyl 2-, 3- and 4-methylpentanoate, and ethyl cyclohexanoate, which exhibit pleasant strawberry-liquorices-like odors. In the early nineties, Takeoka et al. published different papers studying the odor properties of several unsaturated, branched and cyclic esters, including the four above mentioned [14,15]. The authors concluded that these ethyl esters possess remarkably low odor thresholds, ranging from 0.001 to 0.01 μg l^{-1} (in water), well below the odor threshold of their major linear cousin, ethyl hexanoate (1–5 μg l^{-1}) [16]. The presence of three of these com-

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pounds in some other natural foodstuffs has been documented. Ethyl 3-methylpentanoate was first reported by Dumont and Adda in Beaufort cheese [17] and several years later was identified by Cormier et al. [18] in milk affected by *Pseudomonas fragi* bacteria, where ethyl cyclohexanoate was also present. The latter was first identified in rum by Heide et al. [19] and then in refined (virgin quality) olive oil by Guth and Grosh [20]. As for ethyl 4-methylpentanoate, Van der Wal et al. [21] found this compound in roasted cocoa more than thirty years ago whereas Ong and Acree [22] reported its presence in both fresh and canned lychee fruit (*Litchi chinesis*) in the late nineties. More recently, Steinhaus and Schieberle [23], and Fritsch and Schieberle [24] have identified ethyl 4-methylpentanoate in dried hop cones (*Humulus lupulus*) used by the brewing industry and in Bavarian Pilsner-type beers, respectively.

However, and in spite of the aroma potential of this group of compounds, their exact sensory role remains for the most unknown because of the lack of analytical data and analytical methods for their determination. To our knowledge, only Guth and Grosch [20], and Fritsch and Schieberle [24] have quantified ethyl 4-methylpentanoate in olive oil and beer samples, respectively, by means of a stable isotope dilution essay.

Solid-phase extraction (SPE) is widely used in analytical laboratories for either sample extraction or sample clean up procedures. Many benefits of SPE methods have been commonly cited including its robustness, potential for automation, capacity for providing clean extracts, selective isolations and even a fractionation of the different sample components. For these reasons, SPE is a powerful pre-concentration technique which can be easily adapted for routine analysis and, in fact, many studies based on SPE procedures for monitoring different compounds in wine samples have been published in the last years [25–28]. However, as the SPE systems have a low number of chromatographic plates [27] the selectivity (measured as the ratio between the chromatographic retention factors of analytes and interferences) must be high in order to get good separations. Such selectivity can be easily achieved if the target molecule is strongly non-polar, such as TCA [28], or if it is rather polar, such as furaneol [29], but in the case that both analytes and interferences are ethyl esters, the SPE system cannot provide a sufficient separation. The outcome is that even if polar interferences, such as fusel alcohols or fatty acids, have been removed, the chromatographic profile is still too complicated to get an adequate mass spectrometrical (MS) signal of compounds present at very low level (ng l^{-1}) if they elute in the section of the chromatogram where most major esters and non-polar interferences are also found.

Additional selectivity can then be provided by multidimensional gas chromatography (MDGC) or by tandem mass spectrometry (MS–MS). While the later is particularly useful if the target compounds have abundant high mass ions, the former should be preferred if analytes are small molecules with relatively poor and unspecific MS fragmentation patterns. An additional advantage of heart-cutting multidimensional gas chromatography (GC–GC) is that it is possible to work in conditions near to mass overload (including large volume injection of a relatively concentrated extract) in the first chromatographic column and still obtain a perfect chromatographic separation in the second analytical column, so that method detection limits can be improved [30]. MDGC techniques have already been used in wine research with different purposes such as quality and authenticity control [31–33], identification of novel aroma compounds [12,34,35] or quantification of trace odorants [36].

The main purpose of the present paper is, therefore, the development and validation of an analytical method based on selective SPE and further multidimensional gas-chromatographic analysis for the determination of four novel ester compounds in wine and other alcoholic beverages. A second objective of this work is to assess the potential importance of these compounds in the aroma and flavor of different wines and alcoholic beverages.

2. Materials and methods

2.1. Chemicals and reagents

Dichloromethane, methanol, and ethanol, LiChrosolv quality, were from Merck (Darmstadt, Germany). Pure water was obtained from a Milli-Q purification system (Millipore, Bedford, MA, USA). Polypropylene cartridges (3 ml) prepacked with LiChrolut EN resins were also obtained from Merck whereas $NaHCO_3$ was supplied by Panreac (Barcelona, Spain). The chemical standards used for quantitative analysis were from Lancaster (Frankfurt, Germany) or Sigma (St. Louis, MO, USA). The internal standard solution contained 4-hydroxy-4-methyl-2-pentanone at 1500 μg ml^{-1} in dichloromethane.

2.2. Samples

A total of 31 samples were analyzed. This set included wines submitted to biological and/or oxidative aging from Andalusia (Southern Spain), fortified Porto wines (Portugal), wines made with grapes affected by *Botrytis Cinerea* (noble rot) from Sauternes (France) and Tokaji (Hungary), aged red wines, young red and white wines from northern Spain and a Cava (Spanish sparkling wine). Four distilled alcoholic spirits, two Sherry brandies and two Scotch whiskeys, were also included in this study. All the samples were purchased from a wine-retailer in Zaragoza. Some details on the sample origins, aged or alcoholic content are given in Table 1.

Wine Pazo Ribeiro (sample 3 in Table 1), virtually free of the analytes (only 14 ng l^{-1} of 4-methylpentanoate were found), was used for validation purposes.

2.3. SPE modelling and optimization

Solid–liquid distribution coefficients of the four analytes between LiChrolut EN resins and wine or different rinsing systems were measured as explained in [27]. For these measurements, a Saturn 2200 GC-Ion Trap MS from Varian (Walnut Creek, CA, USA) was employed using standard conditions. Chromatographic retention factors were calculated from the corresponding solid–liquid distribution coefficients with the expression $k = K_{sl} \times \phi$; being ϕ the phase ratio in the SPE bed. This was defined as m_o/V_m, being m_o the mass of sorbent in the bed, and V_m the dead volume. For a 200 mg bed filled with