# RESEARCH NOTE
# The Olfactory Threshold of Rotundone in Brandy is Ten-fold Higher than in Wine and does not Increase with the Complexity of the Matrix

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**Rotundone is the only known aroma compound imparting a peppery aroma to wine. Its presence has recently been documented in spirits, notably those aged in oak barrels. However, the sensory contribution of rotundone in such alcoholic beverages remains hard to predict, given their high ethanol level. The aim of this study was to estimate olfactory thresholds for rotundone in brandy using three different matrices. Rotundone concentrations in the unspiked samples were 50 ng/L for wine distillate and 135 ng/L for brandy, demonstrating for the first time the presence of rotundone in such spirits and a possible endogenous origin. The olfactory detection threshold was estimated at 103 ng/L in 40% (v/v) ethanol solution, while difference thresholds were 171 ng/L and 189 ng/L in fresh wine distillate and young French brandy, respectively, both standardised at 40% (v/v). These thresholds were 10-fold higher than in wines and did not significantly differ according to the complexity of the matrix. Our results, which are still preliminary and would deserve to be validated with a larger number of samples and using a higher number of panellists, open new fields of investigation for a deeper exploration of the concentration range of rotundone in brandies, particularly those that underwent a longer ageing period in barrels.**

## INTRODUCTION

Rotundone is the only known aroma compound responsible for peppery notes in wine (Wood *et al*., 2008). This compound has been identified in red wines made from Syrah/Shiraz, Duras, Gamay, Schioppettino or Vespolina (Wood *et al*., 2008; Mattivi *et al*., 2011; Geffroy *et al*., 2014, 2016). Rotundone concentrations do not exceed 150 ng/L (Geffroy *et al*., 2020) except in wines produced under cool and prevailing wet climate conditions, notably temperatures below 25°C during maturation (Takase *et al*., 2015; Zhang *et al*., 2015) or made from grapes infected by powdery mildew (*Erysiphe necator*), in which it can reach up to 400 ng/L (Geffroy *et al*., 2015).

A recent study highlighted the presence of rotundone in French and American oak-aged spirits, including bourbon, whiskey, rum and tequila, with concentrations up to 1 350 ng/L (Genthner-Kreger & Cadwallader, 2021). In this latter study, an increase in rotundone was noted with ageing time in oak barrels, suggesting that it may originate from oak wood. This hypothesis is particularly plausible, given that rotundone has been found in several plant or tree species (Ishihara *et al*., 1991; Wood *et al*., 2008), and that the extraction of this hydrophobic compound might have been enhanced by the high ethanol content of spirits (Caputi *et al*., 2011). Rotundone was also found at 100 ng/L in unaged tequila, indicating that it may also come from agave, and not have been degraded by heat during distillation (Genthner-Kreger & Cadwallader, 2021). Indeed, rotundone is known to be chemically stable in bottled wines after several years of storage (Herderich *et al*., 2012).

From a sensory point of view, rotundone is potent, with an olfactory detection threshold (ODT) established at 8 ng/L in water and 16 ng/L in red wine (Wood *et al*., 2008). Specific anosmia has been reported for rotundone, as 20% to 25% of panellists within the Australian population were not able to detect it even at very high concentrations (Wood *et al*., 2008).

Ethanol concentration is known to have an influence on molecular interaction and flavour portioning (Ickes & Cadwallader, 2018). This means that ODT determined in water or wine cannot be extrapolated to spirits and used to calculate an odour activity value (OAV) with the aim to estimate the contribution of a given aroma compound. An increase in ethanol concentration theoretically induces a decrease in the headspace concentration of aroma compounds as a likely consequence of the increase in their solubility, and therefore leads to an increase in ODT (Athès *et al*., 2004;

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Aznar *et al*., 2004). However, this cannot always be observed practically. Research aiming at determining ODT in spirits or in 40% (v/v) ethanol solutions is scarce (Salo *et al*., 1972; Poisson & Schieberle, 2008; Thibaud *et al*., 2020). The results of these works underline that ODT in such matrices could be either lower or higher in comparison to in a wine or model wine. For example, ODT for 2-phenylethanol was estimated at 2 600 µg/L and 10 000 µg/L in a 40% and 10% (v/v) ethanol solution, respectively (Guth, 1997; Poisson & Schieberle, 2008). In contrast to the above, the ODT of several monoterpenols was higher in a spirit simulant at 40% (v/v) than in wine (Thibaud *et al*., 2020).

The aim of this work was to estimate the ODT of rotundone in brandies and to investigate the effect of three matrices, viz., ethanol solution (40% (v/v), wine distillate and brandy, on these thresholds. Although the presence of rotundone had never been quantified in brandies, it is likely that this compound may be present, as brandies are made through the distillation of wine and are aged in oak barrels.

## MATERIALS AND METHODS

### Wine distillate, brandy and ethanol solution

White wine (Ugni blanc) produced in 2021 was double distilled in a pot still (alembic) and produced on an industrial scale one month before performing the sensory evaluation. The brandies used were purchased from a local shop (Toulouse, France) and had undergone a two-year ageing process in barrels made from French oak wood (*Quercus robur* and *Quercus petrae*). The brandies were selected by a group of experts based on the absence of any peppery aroma at tasting, and for being typical of those produced in the area. During the olfactory examination, the wine distillate was characterised by intense floral and fruity notes, while the brandy was more complex and exhibited fruity and toasted aromas. The ethanol content of both samples was determined using a DMA 5000 M densitometer with an uncertainty of 0.01% (v/v) (Anton Paar, Graz, Austria). It was standardised at 40% (v/v) using ultrapure water obtained from a MilliQ integral 15 system (Merck, Darmstadt, Germany) before the sensory tests and rotundone determination were performed.

An ethanol solution at 40% (v/v) was prepared using absolute ethanol (purity > 99.8%) provided by Sigma-Aldrich (Saint-Quentin-Fallavier, France), and ultrapure water obtained using the previously described equipment. The pH was adjusted to 4.00 using a 0.01 M solution of sulphuric acid.

### Determination of rotundone

The determination of rotundone was performed by stir bar sorptive extraction (SBSE) with heart-cutting two-dimensional gas chromatography mass spectrometry (GC-GC-MS) using the previously described parameters (Takase *et al*., 2015), and *d5*-rotundone (purity > 95%) provided by Eptes (Vevey, Switzerland). Food-grade rotundone (purity > 99%) provided by Firmenich (Geneva, Switzerland) was used to spike the samples. The spiked and control samples were prepared 24 h before the sensory sessions for equilibration of the headspace in testing bottles. The results of the rotundone determinations using the previously cited method confirmed that the concentration of added rotundone was appropriate, with a recovery rate of 79 to 87 %. For all the tasting sessions, samples were coded with three-digit codes, and a volume of 10 mL was poured at 18 °C in black wine-tasting glasses. The tasting sessions took place in a neutral room with white walls and the spacing between the panellists ensured that no communication could occur. All ethanol and rotundone determinations were carried out in duplicate.

### Determination of olfactory detection thresholds (ODT)

The olfactory threshold concentrations of rotundone in 40% (v/v) ethanol solution, wine distillate and brandy were determined during three separate sensory sessions according to the American Society of Testing and Materials method E 679 (ASTM, 2004), which is a rapid method to determine the ODT of rotundone (Wood *et al*., 2008). The sensory panel, which differed according to the matrix being investigated (Table 1), was composed of students from the École d'Ingénieurs de Purpan (EI Purpan) who had little experience with difference testing, and then either of staff from EI Purpan or experts who had greater experience in discrimination tests, or in tasting wine distillates and brandies. Because of these differences in the panel, ASTM E 679, which provides individual thresholds in a unique session, also makes it possible to investigate the impact of experience or origin on the ability to perceive rotundone. The spiked concentrations were chosen based on preliminary tests conducted with a small group of experienced panellists, and previous ODT values reported for rotundone in water and wine (Wood *et al*., 2008; Cullere *et al*., 2016).

The three matrixes, viz., ethanol solution (40% (v/v), wine distillate and brandy, were spiked with rotundone at 5, 10, 20, 40, 80 and 160 ng/L. The panellists who could not detect the highest concentrations of rotundone were retested at 320 and 640 ng/L. Those who could not detect rotundone at 640 ng/L were considered to be anosmic respondents. Previous research showed that 75% to 92% of the panellists who could not detect rotundone at a similar concentration were not able to detect rotundone even at 4 000 ng/L (Wood *et al*., 2008). The three-alternative forced choice (3-AFC) tests, with control samples incorporated, were served in ascending order of rotundone concentrations. Assessments were only conducted orthonasally, with no tasting.

### Statistical analysis

The individual olfactory thresholds were determined for each panellist as the geometric mean of the concentration at which the last miss occurred and the next higher concentration. For all panellists who successfully detected rotundone at a concentration below or equal to 640 ng/L, the subgroup panel threshold was calculated for each matrix as the geometric mean of the individual olfactory thresholds, as described in the E 679 method (ASTM, 2004). For this subgroup, and as the data did not follow a normal distribution, a Mann-Whitney test was performed to detect any differences in threshold according to experience or origin. A Kruskal-Wallis test using a 5% significance level was also performed to investigate whether thresholds might differ according to the type of matrix. These statistical tests were performed using XLSTATS software (Addinsoft, Paris, France).

TABLE 1
Number of panellists by gender, origin and age characteristics for the three panels used for the determination of rotundone olfactory thresholds in 40% (v/v) ethanol solution, wine distillate and brandy.

| Parameters | | 40 % (v/v) EtOH solution (n = 25) | Wine distillate (n = 28) | Brandy (n = 25) |
|---|---|---|---|---|
| Gender | Male | 11 | 14 | 11 |
| | Female | 14 | 14 | 14 |
| Origin | Experts | 14 | 13 | 0 |
| | Students from EI Purpan | 11 | 15 | 17 |
| | Staff from EI Purpan | 0 | 0 | 8 |
| Age (years) | Average age (years) and SD | 27.5 ± 7.2 | 26.4 ± 6.3 | 26.8 ± 7.0 |
| | Minimal age | 21 | 21 | 21 |
| | Maximal age | 45 | 45 | 50 |

## RESULTS AND DISCUSSION

### Rotundone in wine distillate and brandy

The rotundone concentrations quantified in the control samples were 50 ± 3 ng/L and 135 ± 7 ng/L for wine distillate and brandy, respectively, showing for the first time the presence of rotundone in wine distillate and brandy. The results also mean that, for these two matrices, the sensory experiment made it possible to determine *stricto sensus* a difference threshold and not a detection threshold. These notable concentrations in the distillate confirm a possible endogenous origin of rotundone in brandy (Genthner-Kreger & Cadwallader, 2021). However, it should be noted that limited concentrations are expected in brandy base wines before distillation, as rotundone is rarely found at substantial levels in white wines for which the winemaking process involved an early removal of skin, the grape compartment in which rotundone is mainly located (Caputi *et al*., 2011; Geffroy *et al*., 2020). It must also be pointed out that brandy-based wines are made from grapes with a low level of maturity, a condition not favourable for obtaining high levels of rotundone, as this compound is known to accumulate late during maturation (Geffroy *et al*., 2014). High levels of rotundone found in the wine distillate can likely be explained by the concentrating effect of distillation on volatile compounds (i.e., ethanol, aroma molecules), typically by a factor of four or more in the case of brandy distillation. This enables the transformation of a base wine at 9% (v/v) into a distillate at 70% (v/v), and then into a brandy at 40% (v/v) after ageing and/or water addition. The 2021 vintage during which the wine distillate was produced was wet and cool during the maturation period, climatic conditions that are known to enhance rotundone accumulation in grapes (Geffroy *et al*., 2020). It cannot be excluded that low rotundone concentrations would have been found in a wine distillate produced during a warmer and drier season, which tends to be common in France in the context of climate change (van Leeuwen & Darriet, 2016).

The rotundone concentration found in the control brandy samples is in the same range as those previously reported in whiskey or rum aged for at least four years in used American oak barrels (*Quercus alba*) (Genthner-Kreger & Cadwallader, 2021). The fact that this concentration was reached in brandy after only two years `could be the consequence of a high initial rotundone concentration in the unaged spirit, or of differences in the barrels used for ageing (number of refills, origin of the oak wood).

### Rotundone olfactory thresholds

The distribution of the olfactory thresholds for each studied matrix is shown in Fig. 1. The panel thresholds were estimated at 103, 171 and 186 ng/L for 40% (v/v) ethanol solution, wine distillate and brandy, respectively. Despite the general trend towards an increase with the complexity of the matrix, the olfactory thresholds did not differ significantly according to the matrix ($P$ = 0.111). This conclusion is in contradiction with previous results showing an increase in aroma threshold with the increase in the fruity complexity of the matrix for $\beta$-damascenone (Pineau *et al*., 2007). However, as $P$ was not far from the 0.05 threshold, it cannot be ignored that significant results may have been obtained using a larger number of panellists.

The results that highlight a 10-fold difference in olfactory threshold in comparison to wine are similar to observations made for monoterpenes (Thibaud *et al*., 2020). However, in the latter study, the difference was limited to a factor of two to four. The inability of certain panellists to detect rotundone in brandies might be related to the low volatility of the molecule and its hydrophobic character, which that may have limited its headspace concentration (Caputi *et al*., 2011).

OAVs can best be estimated for the wine distillate and brandy by dividing their rotundone concentrations by the ODT determined for the 40% v/v ethanol solution. If rotundone is likely to make a sensory contribution to the aroma of the studied brandy with an OAV of 1.31, it cannot be ignored that rotundone may also contribute to the aroma of wine distillate, despite an OAV of 0.49. It is documented that aroma compounds, and notably terpenes, can have a sensory impact even at subthreshold levels (Poitou *et al*., 2017).

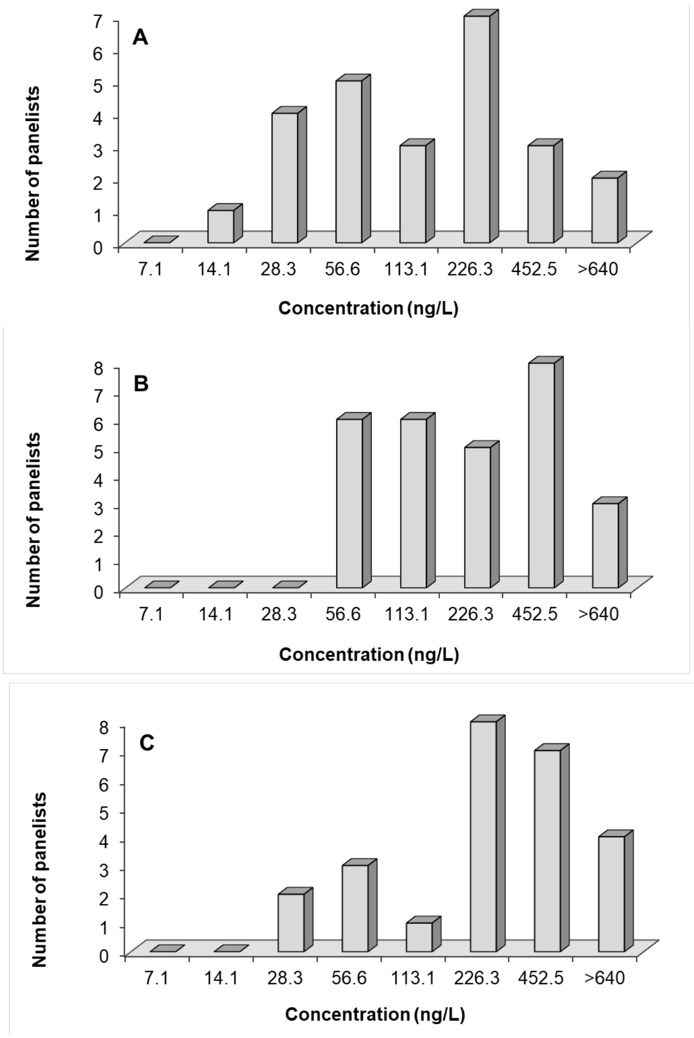


FIGURE 1
Distribution of olfactory thresholds for rotundone in A) 40% (v/v) ethanol solution, B) wine distillate, and C) brandy according to the ASTM E 679 method. The panel thresholds were estimated at 103 ng/L, 171 ng/L and 186 ng/L for 40% (v/v) ethanol solution, wine distillate and brandy, respectively.

The Mann-Whitney test was able to distinguish differences in olfactory threshold according to the origin or experience of the panellists for the three matrices [$P = 0.150$ for 40% (v/v) ethanol solution, $P = 0.890$ for wine distillate, and $P = 0.373$ for brandy], indicating that the diversity of the panels did not introduce any bias in the data.

The percentage of anosmic respondents was 8%, 11% and 16% for 40% (v/v) ethanol solution, wine distillate and brandy, respectively. It must be pointed out that this rate did not differ between the students, staff from EI Purpan and experts (data not shown). These proportions are lower than those previously described by Wood *et al*. (2008). For androsterone, one of the most notable examples of specific anosmia, perception differences were previously demonstrated between European, Asian and Australian populations (Wysocki & Gilbert, 1989). The hypothesis that the rate of specific anosmia to rotundone might differ between French and Australian panellists is unlikely. Indeed, the proportion of less-sensitive subjects determined using 3-AFC tests (water alone vs 200 ng/L water solution), a simplified procedure that is likely to underestimate the proportion of anosmic panellists as one third of the panelists who succeeded in identifying the sample spiked with rotundone are likely to have identified it randomly, reached 31% and even 42% in previous studies conducted on French territory (Geffroy *et al*., 2018, 2020).

Age plays an important role in the ability of panellists to detect aroma compounds with previously documented specific anosmia, including rotundone, with a decline observed after 50 or 55 years (Geffroy *et al*., 2018; Wysocki & Gilbert, 1989). It cannot be ignored that young students might be less subject to specific anosmia. To investigate this hypothesis, 201 students from EI Purpan with an average age of 20.4 ± 0.4 years were assessed using the same simplified procedure, viz., a 3-AFC test including water alone and a 200 ng/L rotundone solution. The results confirm this latter assumption, as the percentage of less-sensitive students unable to identify the correct sample was estimated at 10%, a much lower proportion in comparison with the general population (Geffroy et al., 2018, 2020). If specific anosmia is thought to reflect polymorphisms in the olfactory receptor genes (Boesveldt *et al*., 2017), the findings suggest an early age effect on the sensitivity to detect rotundone, which tends to prove the implication of other regulation mechanisms. The fact that the rate of specific anosmia was not high within the subgroups composed of staff from EI Purpan and experts could be related to their experience, and their exposure to rotundone. Indeed, it was shown that repeated exposure could improve the sensitivity of panellists suffering from hyposmia (Tempere *et al*., 2012). This advantage of training needs to be investigated specifically for anosmia and rotundone.

This also raises the question of whether the panel that consisted of young students and experienced panellists with a higher sensitivity to rotundone, as reflected by the low percentage of anosmic respondents, might have contributed to underestimating olfactory thresholds for rotundone in brandy. In the same way, it cannot be ignored that panellists who could not detect rotundone at 640 ng/L and were considered as anosmic respondents would have succeeded at 1 280 ng/L if this level of concentration had been included in the experimental design. Based on previous research, and as discussed previously (Wood *et al*., 2008), this would have been at the most a concern for one panellist for the simulant and wine distillate panels, and two panellists for the brandy panel. It would have involved a maximum increase in olfactory threshold of 10, 12 and 27 ng/L for ethanol solution at 40% (v/v), wine distillate and brandy, respectively. It should also be mentioned that two panellists out of four who failed at 640 ng/L for brandy also failed at 640 ng/L for the least complex simulant matrix, which suggests that, for these subjects, it was a specific anosmia rather than just a hyposmia.

## CONCLUSIONS

The results show the estimation of the olfactory detection threshold for rotundone at 103 ng/L in 40% (v/v) ethanol solution, and the difference thresholds at 171 ng/L and 186 ng/L in wine distillate and brandy, respectively. These values were not significantly different, indicating that the olfactory thresholds do not increase with the complexity of the matrix. However, further work would be necessary to validate these results using a larger number of samples and a larger number of panellists. Rotundone concentrations in the control samples were 50 ng/L for wine distillate and 135 ng/L for brandy, demonstrating for the first time the presence of rotundone and in these spirits.

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