Food Chemistry 162 (2014) 261–263

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Short communication

Microwave-assisted deuterium exchange: The convenient preparation of isotopically labelled analogues for stable isotope dilution analysis of volatile wine phenols

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ARTICLE INFO

Article history:
Received 18 November 2013
Accepted 12 April 2014
Available online 24 April 2014

Keywords:
Volatile phenols
Microwave-assisted deuterium exchange
Isotope labelling

ABSTRACT

This study reports the convenient, low cost, one-step synthesis of labelled analogues of six volatile phenols, guaiacol, 4-methylguaiacol, 4-ethylguaiacol, 4-ethylphenol, eugenol and vanillin, using microwave-assisted deuterium exchange, for use as internal standards for stable isotope dilution analysis. The current method improves on previous strategies in that it enables incorporation of deuterium atoms on the aromatic ring, thereby ensuring retention of the isotope label during mass spectrometry fragmentation. When used as standards for SIDA, these labelled volatile phenols will improve the accuracy and reproducibility of quantitative food and beverage analysis.

1. Introduction

Gas chromatography–mass spectrometry (GC–MS) is a powerful technique for the compositional characterisation of complex samples, for example the qualitative and quantitative analysis of volatile compounds in food and beverages. Quantitative analysis typically involves the use of internal standards to compensate for analyte losses during sample preparation, due to evaporation or degradation. Ideally, the internal standard is a compound as similar as possible to the analyte of interest, but not already present in the sample matrix. Stable isotope labelled compounds make excellent internal standards (Vining, Smythe, & Long, 1981), since they possess physical and chemical properties that are virtually identical to those of the analyte. Analytical methods using stable isotope dilution analysis (SIDA) therefore enable accurate and reproducible quantification. However, labelled standards are not always commercially available. While traditional organic synthesis can be used to prepare labelled standards via the incorporation of one or more deuterium atoms, these methods can be complex, technically challenging, time consuming, laborious and expensive (Vining et al., 1981); particularly for the preparation of highly deuterated standards.

Volatile phenols have been reported as constituents of various foods and beverages. For example, eugenol is responsible for the distinctive aroma of cloves and also contributes to the aroma of cinnamon (Tikunov, de Vos, González Paramás, Hall, & Bovy, 2010); several volatile phenols, including guaiacol and eugenol, have been identified as glycosidically bound constituents of tomato and strawberry (Tikunov et al., 2010; Ubeda et al., 2012); while vanillin, guaiacol, 4-methylguaiacol and eugenol are oak-derived volatiles present in wine and spirits matured in oak barrels (Pino, Tolle, Gök, & Winterhalter, 2012; Spillman, Sefton, & Gawel, 2004). Volatile phenols have also been implicated in a range of off-odours and taints. Guaiacol was identified as the microbial metabolite responsible for a smoky/phenolic taint in chocolate milk (Jensen, Varelis, & Whifield, 2001), while 4-ethylphenol and 4-ethylguaiacol were found to cause off-odours in strawberries infected with leather rot (Jeleń, Krawczyk, Larsen, Jarosz, & Golebniak, 2005). In wine, these last two compounds are associated with the 'horsy', 'medicinal', 'smoky', 'barnyard' attributes indicative of *Brettanomyces*/*Dekkera* spoilage (Boidron et al., 1988; Chatonnet et al., 1992). A range of volatile phenols, including guaiacol, 4-methylguaiacol, 4-ethylguaiacol, 4-ethylphenol and *o*-, *m*- and *p*-cresols, have recently been identified in grapes and wine tainted by bushfire smoke (Kennison, Gibberd, Pollnitz, & Wilkinson, 2008; Parker et al., 2012).

The preparation of d_3-labelled analogues of guaiacol, 4-methylguaiacol and vanillin have been previously reported, via synthetic pathways involving methylation with deuterated methyl iodide (Pollnitz, Pardon, Sykes, & Sefton, 2004; Spillman, Pollnitz, Liacopoulos, Skouroumounis, & Sefton, 1997). However, the isotope label is readily lost from these analogues upon cleavage of the deuterated methoxy functional group, during mass spectrometry fragmentation, limiting the selection of suitable fragments for selective ion monitoring of the standard. Incorporation of

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http://dx.doi.org/10.1016/j.foodchem.2014.04.051
0308-8146/

deuterium on the aromatic ring ensures retention of the isotope label (Hislop, Hunt, Fielder, & Rowan, 2004), but can be difficult to achieve via traditional synthesis. Pollnitz and colleagues synthesised d_4-4-ethylphenol from the acetate of polydeuterated phenol (Pollnitz, Pardon, & Sefton, 2000); but via a protracted synthetic pathway.

Microwave-assisted chemical reactions have gained popularity in the field of organic synthesis since they were first reported in 1986 (Gedye et al., 1986), primarily because they significantly reduce reaction times and offer convenient, one-step procedures (Chappelle, Kent, Jones, Lu, & Morgan, 2002). Microwave-assisted synthesis can also achieve deuterium exchange on the aromatic ring (Dungey, Hayasaka, & Wilkinson, 2011). This study reports the use of microwave-assisted deuterium exchange for the preparation of isotopically labelled volatile phenols.

2. Materials and methods

2.1. General

Reagents were purchased from Sigma–Aldrich (St. Louis, USA), Honeywell Riedel-de Haën (Germany), Fluka (Japan), Chem Supply (Australia) and Scharlau (Spain). ^1H and ^{13}C NMR spectra were recorded with a Bruker spectrometer (Alexandria, Australia) at an operating frequency of 300 MHz. Mass spectra were recorded with an Agilent 6890N gas chromatograph coupled to a 5973 mass selective detector (Palo Alto, CA, USA).

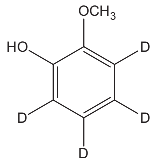
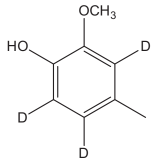
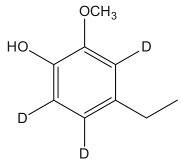
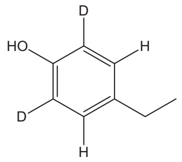
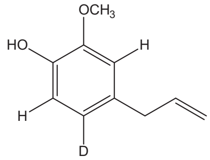
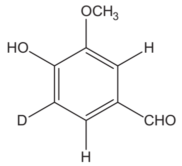
2.2. General procedure for deuterium incorporation into volatile phenols

Approximately 1 g of substrate (Table 1) was suspended in thionyl chloride ($SOCl_2$, 2 mL) and deuterium oxide (D_2O, 20 mL) in the reactor tube of a Discover SP-D microwave apparatus (CEM, Matthews NC, USA). The tube was capped and irradiated at the temperatures and for the durations outlined in Table 1. The reaction mixture was then neutralised with careful dropwise addition of 10% sodium hydroxide solution and extracted with dichloromethane (3 × 50 mL). The combined organic extracts were dried and concentrated, and the crude product purified by Kugelrohr micro-distillation; except for deuterated vanillin which was purified by recrystallisation from D_2O.

2.2.1. [$^2H_4$]-Guaiacol

δ_H ($CDCl_3$): 5.62 (1H, s, OH), 3.88 (3H, s, OCH_3); δ_C ($CDCl_3$): 146.5, 145.5, 55.8; *m*/*z*: 128 (M^+, 87%), 113 (100%), 85 (54%), 57 (12%), 32 (17%), 28 (71%).

Table 1
Experimental conditions and compositional outcomes for microwave-assisted deuterium exchange of volatile wine phenols.

Substrate	Temperature (°C)	Duration (hours)	Isotopic Composition (%)					Structure
			d_0	d_1	d_2	d_3	d_4	
Guaiacol	100	36					**100**	
4-Methylguaiacol	100	30				**100**		
4-Ethylguaiacol	100	36				**100**		
4-Ethylphenol	100	144	4	11	**85**			
Eugenol[a]	80	8	3	22	**42**	33		
Vanillin[a]	100	45	3	40	**57**			

[a] One aromatic proton was fully exchanged, but incorporation of the second deuterium occurred incompletely at the remaining positions.

2.2.2. $[^2H_3]$-4-Methylguaiacol

δ_H ($CDCl_3$): 5.46 (1H, s, OH), 3.87 (3H, s, OCH_3), 2.27 (3H, s, CH_3); δ_C ($CDCl_3$): 146.1, 143.2, 129.4, 55.8, 20.8; *m*/*z*: 141 (M^+, 21%), 126 (18%), 98 (6%), 32 (24%), 28 (100%).

2.2.3. $[^2H_3]$-4-Ethylguaiacol

δ_H ($CDCl_3$): 5.46 (1H, s, OH), 3.88 (3H, s, OCH_3), 2.58 (2H, q J = 7.8, CH_2), 1.22 (3H, t, J = 7.8, CH_3); δ_C ($CDCl_3$): 146.2, 143.4, 136.1, 55.8, 28.4, 15.9; *m*/*z*: 156 (4%), 155 (M^+, 42%), 154 (3%), 141 (9%), 140 (100%), 139 (6%), 125 (9%), 97 (5%), 94 (6%).

2.2.4. $[^2H_2]$-4-Ethylphenol

δ_H ($CDCl_3$): 7.06 (2H, s, ArH), 4.68 (1H, s, OH), 2.59 (2H, q J 7.6, CH_2), 1.204 (3H, t J 7.5, CH_3); δ_C ($CDCl_3$): 153.3, 136.5, 128.8, 28.0, 15.9; *m*/*z*: 124 (M^+, 37%), 110 (20%), 109 (100%), 93 (4%), 79 (9%), 78 (7%).

2.2.5. $[^2H_2]$-Eugenol

δ_H ($CDCl_3$): 6.85 (1H, s, ArH), 6.69 (1H, s, ArH), 5.94 (1H, m, CH), 5.49 (1H, s, OH), 5.08 (2H, m, CH_2), 3.87 (3H, s, OCH_3), 3.31 (2H, d J 6.0, CH_2); δ_C ($CDCl_3$): 146.4, 143.8, 137.7, 131.8, 115.5, 114.2, 111.1, 55.9, 39.8; *m*/*z*: 166 (M^+, 100%), 165 (47%), 151 (31%), 134 (29%), 105 (31%), 93 (20%), 78 (20%).

2.2.6. $[^2H_2]$-Vanillin

δ_H ($CDCl_3$): 9.83 (1H, s, CHO), 7.42 (1H, s, ArH), 6.21 (1H, s, OH), 3.96 (3H, s, OCH_3); δ_C ($CDCl_3$): 191.0, 151.6, 147.1, 127.5, 108.7, 56.1; *m*/*z*: 155 (8%), 154 (M^+, 58%), 153 (100%), 152 (54%), 125 (7%), 110 (10%), 82 (11%), 28 (13%).

3. Results and discussion

Application to 4-methylguaiacol of the conditions employed by Dungey and co-workers to prepare d_4-guaiacol (Dungey et al., 2011) gave 100% incorporation of deuterium into the aromatic ring (Table 1). However, deuterium incorporation was not achieved to the same extent for 4-ethylguaiacol, or even guaiacol, under the same conditions. Following optimisation of the method, deuterium atoms were fully incorporated into the aromatic ring, to give $[^2H_4]$-guaiacol and $[^2H_3]$-4-ethylguaiacol. The NMR spectra of labelled guaiacol, 4-methylguaiacol and 4-ethylguaiacol closely matched their unlabelled analogues, but with the absence of signals for aromatic protons. The degree of deuterium incorporation was confirmed by GC–MS (Table 1).

Only partial deuterium exchange was achieved for 4-ethylphenol, eugenol and vanillin, to give $[^2H_2]$-4-ethylphenol, $[^2H_2]$-eugenol and $[^2H_2]$-vanillin; with the extent of deuterium incorporation again confirmed by NMR and GC–MS. In the case of $[^2H_2]$-eugenol and $[^2H_2]$-vanillin, one aromatic proton was fully deuterium exchanged, but incorporation of the second deuterium occurred incompletely at the remaining positions. Although these compounds did not achieve complete deuteration they were still suitable for use as internal standards for analysis by GC–MS. Eugenol decomposition was observed at reaction temperatures above 80 °C or reaction durations above 8 h, in agreement with a previous study reporting decomposition of eugenol at high temperatures following the addition of hydrochloric acid (Kalpala, Hartonen, Huhdanpaa, & Riekkola, 2003). The same study reported the deuteration of eugenol using only D_2O, but we were unable to reproduce these results. Vanillin polymerised under more forcing reaction conditions.

Microwave-assisted deuterium exchange of several other important oak volatiles, i.e. isoeugenol, furfural and oak lactone, was also attempted using various reaction conditions. However, the neat compounds either polymerised upon addition of thionyl chloride (furfural and isoeugenol) or, in the case of oak lactone, underwent only limited deuterium exchange, with a substantial proportion of the product remaining undeuterated.

4. Conclusion

In the current study, microwave technology has been used to produce deuterium labelled volatile phenols in a convenient, one-step synthesis, with the additional benefit of incorporating the deuterium atoms on the aromatic ring. This method is suitable for making labelled analogues for the identification and quantification of these compounds, which are often found in foods and beverages.

Acknowledgements

The authors gratefully acknowledge Josh Hixson for technical assistance. Anna Crump thanks the Grape and Wine Research and Development Corporation for the provision of a research scholarship.

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