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Research Article

The deuteriation of constituents in olive oil and red wine with Nafion, a polymer supported acid catalyst

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Summary

A procedure for the deuteriation of a large range of aromatic compounds with phenolic and methoxy substituents has been developed using Nafion, a polymer supported acid catalyst. A range of compounds present in red wine and olive oil were deuteriated using these conditions. This procedure provides a facile route for the labelling of homovanillic alcohol, homovanillic acid, syringic acid, syringaldehyde and vanillin. This method is also applicable for the tritiation of these compounds. Copyright © 2001 John Wiley & Sons, Ltd.

Key Words: olive oil; red wine; deuteriation; tritiation; polymer supported acid catalyst; antioxidant

Introduction

Over the last decade the health-promoting benefits of a diet rich in antioxidants has become generally recognized. Numerous *in vitro* studies have shown the phenolics present in olive oil and red wine possess strong radical scavenging activity at least equal in potency with other important dietary antioxidants such as ascorbic acid and α-tocopherol.[1–3] The major phenolic compound present in olive oil is

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hydroxytyrosol (HT) I[4,5] and the major phenolic compound present in red wine is gallic acid II.[3]

However, details on the biological fate of these compounds is lacking, mainly due to their low systemic concentration attained since they are typically present in trace amounts in the diet. One way of increasing analytical sensitivity is by using radiolabelled compounds. Tritium labels can be introduced post-synthetically and high specific activities can be obtained. Due to their high specific activities they can be dosed in small quantities which reflect the amounts ingested in the diet and their analysis is quicker than conventional non-radiometric methods of analysis. Radiolabelled compounds are routinely used in biomedical sciences, however sometimes the labelled antioxidants are typically not commercially available or their synthesis is complex.

We published previously an article which described a simple procedure for the deuteriation of hydroxytyrosol I, gallic acid III and a range of other phenolic compounds with Amberlyst 15, a polymer supported acid catalyst.[6] This work has been integral to tracing the metabolic fate and elucidation of the metabolites of hydroxytyrosol I.[7]

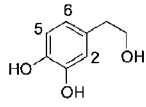
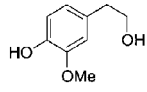
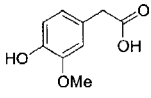
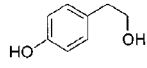
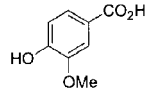
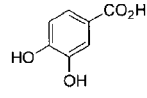
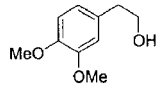
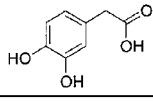
We have recently extended this work to deuteriate a range of compounds with the polymer supported acid catalyst Nafion. The aim of this work was to determine whether the Nafion catalyst or the Amberlyst 15 catalyst gave better incorporation of deuterium with a range of compounds and to investigate whether compounds which had not previously been deuteriated could be labelled with Nafion, specifically two metabolites of hydroxytyrosol I which have been identified recently (homovanillic acid III and homovanillic alcohol IV).[8]

We have deuteriated rather than tritiated the compounds, principally due to the wide availability, high isotopic purity and affordability of deuteriated water and the fact that the reactions can be easily monitored by ^{1}H NMR spectroscopy. Furthermore, the deuteriated analogues can also be used in biological studies where MS is used as the analytical method.

Results and discussion

Previously we optimized a procedure for the deuteriation of phenols[6] with Amberlyst 15 which was a modification of a procedure published by Brewer *et al*.[9] Our modification removed the work-up procedure and the desired compound was obtained after removal of the Amberlyst 15 resin and freeze-drying of the remaining solution. This reaction was

Table 1. Deuteriation of some phenolic constituents related to or present in olive oil[a]

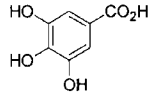
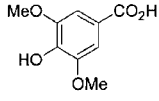
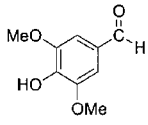
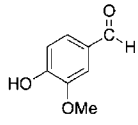
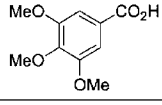
Compound	Structure	Position	% Deuterium incorporation at each site
I		2,6 5	100 30
III		2 5,6	30 60
IV		2 5,6	5 20
V		2,6 3,5	21 77
VI		2,6,5	85[c]
VII		2 5 6	99[b] 99 99
VIII		2 5 6	33 33 100
IX		2,5 6	17 6

[a] Reaction mixture: substrate (~15 mg), Nafion resin (~15 mg) and deuteriated water (0.2 ml). All reactions were carried out at 90°C for 24 h using a Hewlett Packard™ HPLC vial (2 ml capacity) with a magnetic stirrer (7 mm).
[b] Analysis of the NMR solution by HPLC or TLC was required to confirm the substrate had survived the reaction.
[c] Reaction had 1,4-dioxane added as the substrate is insoluble in water and no incorporation occurred under the standard conditions.

simple to perform with no complex glassware required. In addition, the labelling reaction was able to be successfully carried out on as little as 5 mg of substrate. We would like to disclose that a similar procedure can be used with Nafion, another polymer supported acid catalyst.

The results for some phenolic constituents related to or present in olive oil are shown in Table 1, whilst the results for some

Table 2. Deuteriation of some phenolic constituents related to or present in red wine[a]

Compound	Structure	Position	% Deuterium incorporation at each site
II		2,6	100[b]
X		2,6	15[c]
XI		2,6	50
XII		2,5,6	90[c]
XIII		2,6	40

[a] Reaction mixture: substrate (~15 mg), Nafion resin (~15 mg) and deuteriated water (0.2 ml). All reactions were carried out at 90°C using in a Hewlett Packard™ HPLC vial (2 ml capacity) with a magnetic stirrer (7 mm).
[b] Analysis of the NMR solution by HPLC or TLC was required to confirm the substrate had survived the reaction.
[c] Reaction had 1,4-dioxane added as the substrate is insoluble in water and no incorporation occurred under the standard conditions.

phenolic constituents related to or present in Table 1 red wine are shown in Table 2. In all cases, the extent of deuterium incorporation was determined by ^{1}H NMR spectroscopy, with complete disappearance of the signal indicating that the proton had completely exchanged.

It can be seen from Tables 1 and 2 that Nafion can be used for the incorporation of deuterium into a wide range of aromatic compounds with a hydroxy or methoxy substituent. The majority of substrates gave efficient deuterium incorporation with the Nafion catalyst. In three cases the compound (VI, X and XII) were not soluble in warm water, and no incorporation occurred. The addition of

1,4-dioxane solved this problem and efficient incorporation subsequently occurred.

From this study with Nafion and our previous study with Amberlyst 15 we have come to several conclusions about the use of polymer supported acid catalysts. Amberlyst 15 is more economical than Nafion. However, Nafion is simpler to use with only one crystal required, this decreases the surface area of the reaction but makes the work up of the reaction simpler. Nafion as the acid catalyst gave superior deuterium incorporation in the compounds V, VI and XIII, although Amberlyst 15 gave superior incorporation of deuterium in compounds I, VIII and IX.

The percentage of deuterium incorporation was dependent on the functional groups on the aromatic ring. From these studies and the previous study it can be stated that only the protons *ortho* and *para* to hydroxyl groups will exchange with certainty. The hydroxyl groups mesomerically activate the *ortho* and *para* positions and the protons in these positions will exchange preferentially; this is in accordance with an electrophilic substitution type mechanism. Methoxy groups also mesomerically activate the *ortho* and *para* positions of the aromatic ring, however they are not as strongly electron withdrawing as a hydroxyl group and hence the incorporation will not be as efficient.

In several compounds the incorporation of deuterium was only 10–20%, however, even this relatively low degree of incorporation should provide a product with adequate isotopic abundance for pharmacokinetic and metabolism studies.

We have demonstrated that the compounds homovanillic alcohol III, homovanillic acid IV, syringic acid X, syringaldehyde XI, and vanillin XII, can be labelled by this method. A method for their labelling has not been previously published.

In conclusion, we have shown that heterogeneous acid-catalysed H/D exchange can be used for the incorporation of a deuterium label in a number of antioxidant compounds which are present in olive oil and red wine. This procedure has also been used for the deuteriation of homovanillic alcohol III and homovanillic acid IV which are two metabolites of the chief phenolic antioxidant in olive oil. Availability of labelled analogues of the latter compounds will aid in determining the factors responsible for modulating the disposition and metabolism of the parent phenolic antioxidant.

Experimental

Materials

The substrates were used as received from Sigma–Aldrich, except for hydroxytyrosol I which was synthesized from 3,4-dihydroxyphenyl acetic acid IX by the procedure of Capasso *et al.*[10] Deuterium oxide (99.9%) was used as received from Aldrich. The Nafion NR50 beads were available commercially from Aldrich.

Hydrogen isotope exchange reaction

The substrate ($\sim$15 mg), Nafion (1 bead, $\sim$15 mg), deuterium oxide (0.2 ml), and a magnetic stirrer (7 mm) were introduced into a Hewlett Packard™ HPLC vial (2 ml capacity). The flask was evacuated with N_2, the top was screwed on, and the flask was placed in an oil bath. The reaction vial was heated at 90°C, with continuous stirring, for the desired reaction time (typically 24 h). On completion the tube was cooled, the solution removed and water (0.2 ml) was added to the flask to rinse the resin; this solution was removed and added to the initial solution. This procedure was repeated twice, to ensure complete removal of the substrate. The recovered substrate was obtained after the combined solutions were freeze-dried.

In the cases where the substrate was not soluble in cold water, diethyl ether (2×0.2 ml) was added to the reaction vessel instead of water, to ensure complete removal of the substrate from the resin. The diethyl ether was removed in *vacuo* and the residual solution was freeze-dried.

The regiospecificity of the deuteriation was determined by ^{1}H NMR spectroscopy on a Varian spectrometer, with an operating frequency of 300 MHz. ^{1}H resonances are quoted in parts per million downfield from the ^{1}H resonance of tetramethylsilane (TMS). Depending on the solubility of the substrate *d*-chloroform, d_6-acetone or d_2-water were used as the solvents. In the cases when all the protons were completely exchanged analysis of the NMR solution by HPLC or TLC was required to confirm that the substrate had survived the reaction conditions.

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