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The effect of 2-hydroxypropyl *β*-cyclodextrin on the stability of polyphenolic compounds from *Moringa oleifera* Lam leaf extracts in a natural low-transition temperature mixture

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HBA, hydrogen bond acceptor
HBD, hydrogen bond donor
HP-*β*-CD, 2-hydroxypropyl *β*-cyclodextrin
LTTM, low-transition temperature mixture
MoL, *M. oleifera* leaves

Abstract

Polyphenol extracts from *Moringa oleifera* leaves (MoL) were obtained with a glycerol-based low-transition temperature mixture (LTTM) and a combination of LTTM with 2-hydroxypropyl *β*-cyclodextrin (HP-*β*-CD). The extracts were maintained at 4, 22 and 50 °C for 18 days and the antiradical activity (A_{AR}) was recorded to detect modifications in the antioxidant activity of the extracts. A_{AR} displayed a constant decline at every temperature tested, following pseudo first-order kinetics and the decay constants suggested that the presence of HP-*β*-CD had a protective action, slowing down A_{AR} decline. The analysis of the polyphenolic profiles using liquid chromatography-diode array-mass spectrometry revealed that after storage for 18 days at 50 °C, the major quercetin glycosides occurring in MoL were extensively degraded. Based on the detection of protocatechuic acid in the stored extracts, putative pathways of flavonol glycoside degradation were proposed. It was concluded that the decomposition of these components was mainly responsible for the A_{AR} decline observed.

Introduction

Plants have played a central role in maintaining human health for millennia. Herbs have been widely used all over the world as food and medicines and nowadays research has focused on various botanicals that possess antitumor, anti-inflammatory and immune-stimulating properties, which may contribute in reducing the risk of cardiovascular disease and cancer. In different herbs, a broad variety of active phytochemicals, including simple phenolics and flavonoids have been identified as the major active constituents and numerous methodologies have been developed for the effective preparation of polyphenol-containing extracts.

Moringa oleifera leaves (MoL) is a tissue that displays several bioactivities beneficial to human health (Farooq *et al.* 2012) and possess a relatively high richness in bioactive polyphenols (Lalas *et al.* 2017). For this reason, there have been some efforts pertaining to the efficient polyphenol extraction, by employing green extraction media, such as water/ethanol mixtures (Wang *et al.* 2017).

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However, a more recent study demonstrated a particularly high effectiveness in polyphenol extraction, using a novel, natural low-transition temperature mixture (LTTM), composed of glycerol and sodium acetate (Karageorgou *et al.* 2017). Following examinations also demonstrated that combination of this LTTM with hydroxypropyl *β*-cyclodextrin (HP-*β*-CD) may boost extraction, achieving even higher yields (Karageorgou *et al.* 2018).

An important issue associated with extraction of natural substances is their stability in the extraction medium over time. Since many polyphenols are inherently unstable molecules, owed to their susceptibility to oxidation, the examination of their stability under specific conditions is imminent and requires detailed examination. On such a ground, this examination was undertaken to investigate the stability of MoL polyphenols in extracts generated using the above-mentioned extraction medium, composed of the LTTM and HP-*β*-CD. Stability in the presence and absence of HP-*β*-CD was assessed by monitoring the antiradical activity (A_{AR}) of the extracts over 18 days at various temperatures, and polyphenol transformations that might be linked with changes in A_{AR} were identified using liquid chromatography-diode array-mass spectroscopy.

Experimental

Plant material

Dried and pulverised *Moringa oleifera* leaves (MoL), with average particle diameter of 0.5 mm, were used. Collection and handling of the plant material have been previously described in detail (Karageorgou *et al.* 2017). All treatments were repeated at least twice.

Chemicals and reagents

Glycerol (99 %), 2-hydroxypropyl *β*-cyclodextrin (98 %) and ethanol (99.8 %) were from Acros Organics (Geel, Belgium). Anhydrous sodium carbonate was from Carlo Erba Reactifs (Val de Reuil, France). Sodium acetate trihydrate was from Penta (Prague, Czeck Republic). Folin-Ciocalteu reagent was from Fluka (Steinheim, Germany). 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH) was from Aldrich (Steinheim, Germany). All solvents used for liquid chromatography were HPLC grade.

Preparation of the LTTM

The LTTM used was prepared using the optimised conditions described previously (Karageorgou *et al.* 2017). Glycerol (HBD) was mixed with an appropriate amount of sodium acetate (HBA) to give a molar ratio HBD:HBA of 6:1, and the mixture was moderately heated under stirring until a transparent liquid was formed. Aqueous solution 80 % (w/v) of this LTTM was used for the extractions.

Batch extraction procedure and sample handling

Extraction of MoL polyphenols was performed with a previously developed optimized methodology (Karageorgou *et al.* 2018). Accurately weighted 2.5 g of dried powdered material was mixed with 100 mL of 80 % (w/v) aqueous LTTM containing 1 % (w/v) 2-hydroxypropyl *β*-cyclodextrin (HP-*β*-CD), to give a liquid-to-solid ratio of 40 mL g^{-1}. Extractions were carried out at 50 °C, under continuous stirring at 600 rpm, for 180 min. Extractions without HP-*β*-CD were also performed, under the same conditions. Following extraction, samples were centrifuged in a table centrifuge (Hermle, Wehingen, Germany) at 10,000 × *g* for 10 min, and the clear extract was used for all subsequent procedures.

Stability test

The centrifuged extracts were divided into 30-mL portions in screw-cap glass vials and stored in a freezer (4 °C), in a dark temperature-controlled chamber (22 °C) and in a thermostated water bath (50 °C). Sampling was carried out at regular intervals, within 18 days.

Determinations

Total polyphenols were determined with the Folin-Ciocalteu methodology and expressed as gallic acid

equivalents. Antiradical activity (A_{AR}) was estimated with the DPPH and expressed as μmol DPPH per g dry weight (Paleologou *et al.* 2016).

Qualitative liquid chromatography-diode array-mass spectrometry (LC-DAD-MS)

For the analyses, a Finnigan MAT Spectra System P4000 pump, a UV6000LP diode array detector and a Finnigan AQA mass spectrometer were employed. Chromatography was performed on a Fortis RP-18 column, 150 × 2.1 mm, 3 μm, at 40 °C. Details of the analytical procedure were given elsewhere (Paleologou *et al.* 2016).

Statistics

At least two experiments were carried out for the extractions and stability tests. Analyses were performed in triplicate and values given are means. Linear regressions were established at least at a 95 % significance level. For all statistics, Microsoft Excel™ 2010 and SigmaPlot™ 10 were used.

Results and Discussion

Kinetics of A_{AR} evolution and the effect of HP-β-CD

To appraise stability of the extracts at various temperatures and to illustrate the role of HP-β-CD, A_{AR} evolution was traced over 18-days period. A_{AR} was chosen as a safe criterion to detect modifications of extracts status, as it is linked with polyphenol structure (Huang *et al.* 2005) and therefore it was hypothesised that any polyphenol modification would be readily reflected on the A_{AR}. It was revealed that A_{AR} showed a declining tendency, described by first-order kinetics (Eq. 1):

$$A_{AR(t)} = A_{AR(0)}\, e^{-kt} \quad (1)$$

where $A_{AR(t)}$ is the A_{AR} at any time t; $A_{AR(0)}$ the initial A_{AR} value and k the first-order decay constant. The linearised form of Eq. 1 is given as follows (Eq. 2):

$$ln(A_{AR(t)}) = ln(A_{AR(0)}) - kt \quad (2)$$

Rearrangement of Eq. 2 would give (Eq. 3):

$$ln\left(\frac{A_{AR(t)}}{A_{AR(0)}}\right) = -kt \quad (3)$$

Thus k (days^{-1}) could be determined graphically from the slope of the straight line obtained after plotting $ln\left(\frac{A_{AR(t)}}{A_{AR(0)}}\right)$ against t. The half-life of A_{AR} decrease could then be estimated as shown below (Eq. 4):

$$t_{½} = -\frac{ln(0.5)}{k} \quad (4)$$

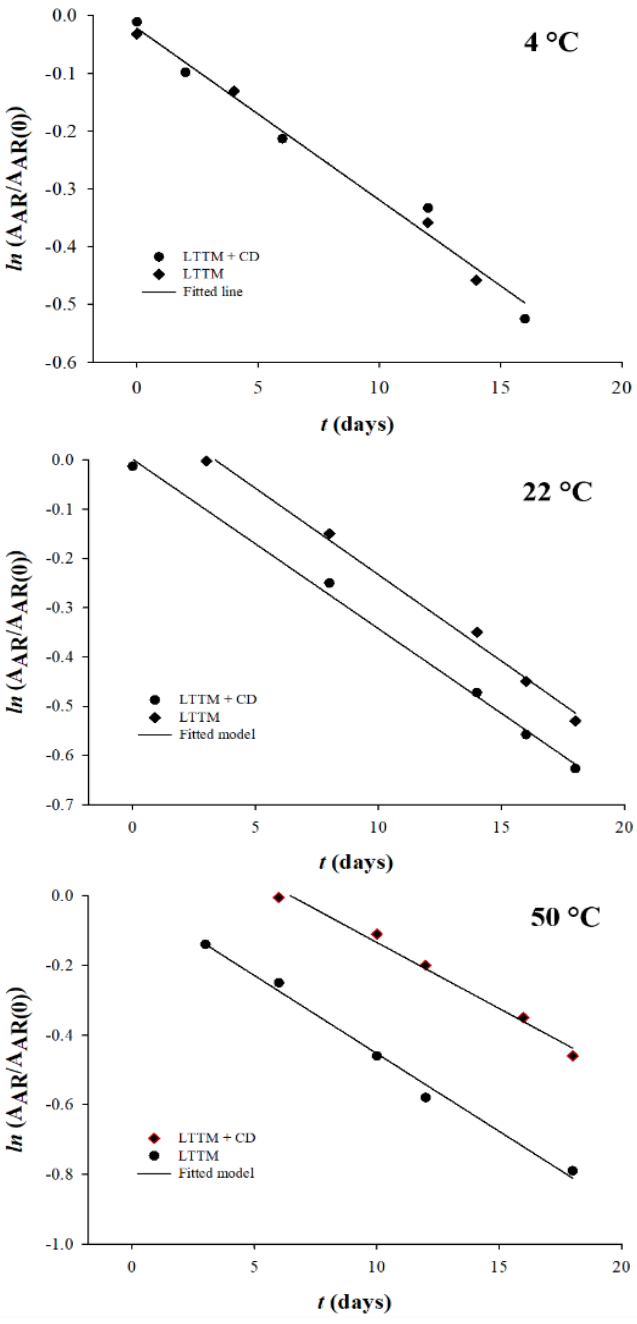

Fig. 1. First-order kinetics of A_{AR} decay in MoL extracts stored at 4, 22 and 50 °C. LTTM with (rectangles) and without 2-hydroxypropyl β-cyclodextrin (CD) (circles).

Table 1. Kinetic parameters estimated for A_{AR} decay in MoL extracts.

Solvent	Temperature [°C]					
	4		22		50	
	k	$t_{½}$	k	$t_{½}$	k	$t_{½}$
LTTM + CD	0.0297	23.3	0.0344	20.1	0.0379	18.3
LTTM	0.0297	23.3	0.0351	19.7	0.0448	15.5

As can be seen in Fig. 1 and Table 1, kinetics at 4 °C was identical for extracts stored either in the presence or in the absence of HP-*β*-CD. However, differentiations appeared upon storage at 22 and 50 °C, where A_{AR} exhibited apparently higher stability in the presence of HP-*β*-CD. From the data given in Table 1, it was illustrated that the pseudo-first order decay constant for the A_{AR} increased by almost 22 % from 4 to 50 °C, in the extracts containing HP-*β*-CD. To the contrary, the increase in the extracts lacking HP-*β*-CD was approximately 34 %. This outcome highlighted the role of HP-*β*-CD in A_{AR} stability.

Bibliographic data on polyphenol stability in DES are scarce and therefore no concrete conclusions could be drawn. Regarding green tea polyphenols, it was recently demonstrated that they had better stability in a DES than in conventional solvents, including aqueous mixtures of methanol and ethanol (Jeong *et al.* 2017). No clear explanation for such a phenomenon has been provided, but it could be presumed that hydrogen bonds developed between polyphenols and the HBA could act in a stabilizing manner, since polyphenols may act as HBDs (Bi *et al.* 2013). However, polyphenol instability in a medium such as the DES used in this study may stem from the intrinsic basicity of the solvent, owed to the presence of the acetate anion. At this point, the presence of HP-*β*-CD might be crucial. Quercetin glycosides and other flavonol aglycones may form complexes with cyclodextrins, such as *β*-cyclodextrin (*β*-CD) and HP-*β*-CD (Alvarez-Parilla *et al.* 2005; Jullian *et al.* 2007; Lucas-Abellán *et al.* 2008) and it has been demonstrated that flavonoid/HP-*β*-CD complexes provided improved stability of the encapsulated molecule, as opposed to the free (non-encapsulated) one (Nguyen *et al.* 2013). Such a finding was ascribed to the protective effect of HP-*β*-CD cavity (Liu *et al.* 2006). Studies on polyphenol-containing extracts (Mourtzinos *et al.* 2008; Kalogeropoulos *et al.* 2010) were in the same line.

Investigations on rosmaric acid inclusion complexes by various cyclodextrins (Çelik *et al.* 2011) gave an explanation concerning the correlation of polyphenol stability with enhanced antioxidant activity. It has been proposed that once a polyphenol radical is formed and engulfed in HP-*β*-CD hydrophobic cavity, it could be better stabilised through resonance, by intramolecular hydrogen bonding. As a consequence, the redox potential between the aroxyl radical and the reduced polyphenol may be lowered, endowing the polyphenol higher antioxidant activity. Indeed, several examinations showed that polyphenols such as chlorogenic acid (Shao *et al.* 2014), quercetin and rutin (Alvarez-Parilla *et al.* 2005), rosmarinic acid (Çelik *et al.* 2011; Medronho *et al.* 2014), and quercetin and glycosides therof (Çelik *et al.* 2015), encapsulated in cyclodextrins may behave as more potent antioxidants, compared with the non-encapsulated molecules. Thus, the slower A_{AR} decay recorded for MoL extracts in the presence of HP-*β*-CD could be owed to effective inclusion and higher polyphenol stability.

Modifications in the polyphenolic profile

To ascertain if alterations in the polyphenolic profile were responsible for the changes in A_{AR} observed, LC-DAD-MS was performed for the samples stored at 50 °C, which had the highest decay rate. In the chromatogram traced at 340 nm for the initial extract obtained with the LTTM/HP-*β*-CD (Fig. 2, upper chromatogram), eight polyphenolic substances could be tentatively identified. The extract obtained only with the

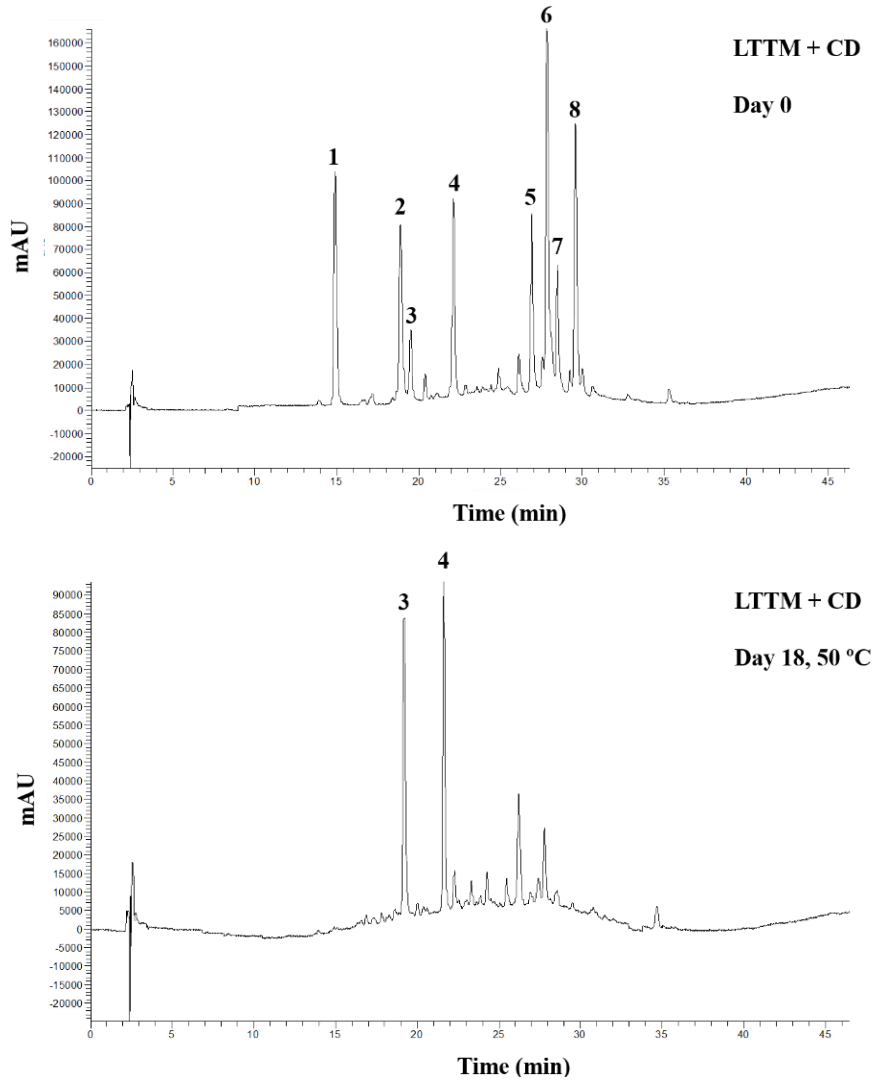

Fig. 2. Chromatogram of MoL extracts before (day 0) and after storage at 50 °C (day 18). The chromatograms were monitored at 340 nm. Peak assignment is as in the Table 2.

LTTM (no HP-*β*-CD) had identical profile (data not shown). This was confirmation of previous results, which demonstrated that HP-*β*-CD acted merely by boosting extraction yield. Based on published data, peak #1 was identified as chlorogenic acid, while peaks #2 and 3 as chlorogenate derivatives (Table 2). Peak #4 was assigned to multiflorin B, peaks #5 – 7 to quercetin glycosides and peak #8 to a kaempferol glycoside (Karageorgou *et al.* 2017). When the extracts were analysed after 18 days of storage at 50 °C, it was revealed that the polyphenolic profile was fundamentally altered (Fig. 2, lower chromatogram). Peaks #1 and 2 completely disappeared, while peaks #5 – 8 suffered extensive degradation. However, this modification did not result in glycoside hydrolysis, as no quercetin or kaempferol aglycones were detected. This finding led to the conclusion that decomposition of these substances would have involved cleavage of the flavonoid skeleton.

To ascertain such a hypothesis, chromatograms were also recorded at 280 nm, to detect the formation of compounds that could have emerged through quercetin or kaempferol degradation. Indeed, two products associated with quercetin oxidative degradation were detected, protocatechuic acid (PA) and the depside 2-(3,4-dihydroxybenzyloxy)-4,6-dihydroxybenzoic acid, termed as quercetin oxidation product (QOP) (Fig. 3). These compounds did not occur in the initial (untreated) extract and consisted sound evidence of quercetin decomposition. In fact, PA may derive from quercetin *via* thermal degradation (Makris and Rossiter 2000), free-radical oxidation (Makris and Rossiter 2002) and enzyme-mediated oxidative cleavage (Osman *et al.* 2008). However, the generation of QOP would require the existence

Table 2. UV-vis and mass spectrometry data for the main polyphenols and some degradation products thereof, detected in the extracts of *M. oleifera* leaves.

Peak	Rt [min]	UV-vis	$[M + H]^+$ [*m/z*]	Other ions (*m/z*)	Tentative identity
1	14.90	244, 318	355	377$[M + Na]^+$, 163	Chlorogenic acid
2	18.88	246, 320	513	427, 377, 355, 163	Chlorogenate derivative
3	19.50	242, 318	703	733$[M + H + MeOH]^+$, 695, 545, 409	Chlorogenate derivative
4	22.12	270, 340	595	617$[M + Na]^+$, 457, 379	Apigenin rutinoside (multiflorin B)
5	26.90	256, 356	465	487$[M + Na]^+$, 303	Quercetin glucoside or galactoside
6	27.82	256, 360	551	573$[M + Na]^+$, 303	Quercetin glycoside
7	28.46	266, 346	877	791, 551, 303	Quercetin rhamnoside derivative
8	29.58	264, 350	535	577$[M + Na]^+$, 287	Kaempferol malonylglucoside
PA	12.38	258, 292	153*	-	Protocatechuic acid
QOP	15.73	260, 296	305*	153	Quercetin oxidation product

* Negative ionisation

of a quercetin dioxygenated derivative and therefore it was suspected that the oxidative degradation followed the pathway proposed for room temperature quercetin autoxidation (Zenkevich *et al.* 2007). Initially, quercetin hydrolysis may occur giving rise to quercetin aglycone. Following this step, quercetin radicals may be formed, most probably due to the presence of transition metal ions (e.g. Fe or Cu), which then may react with the di-radical O_2 (Fig. 4).

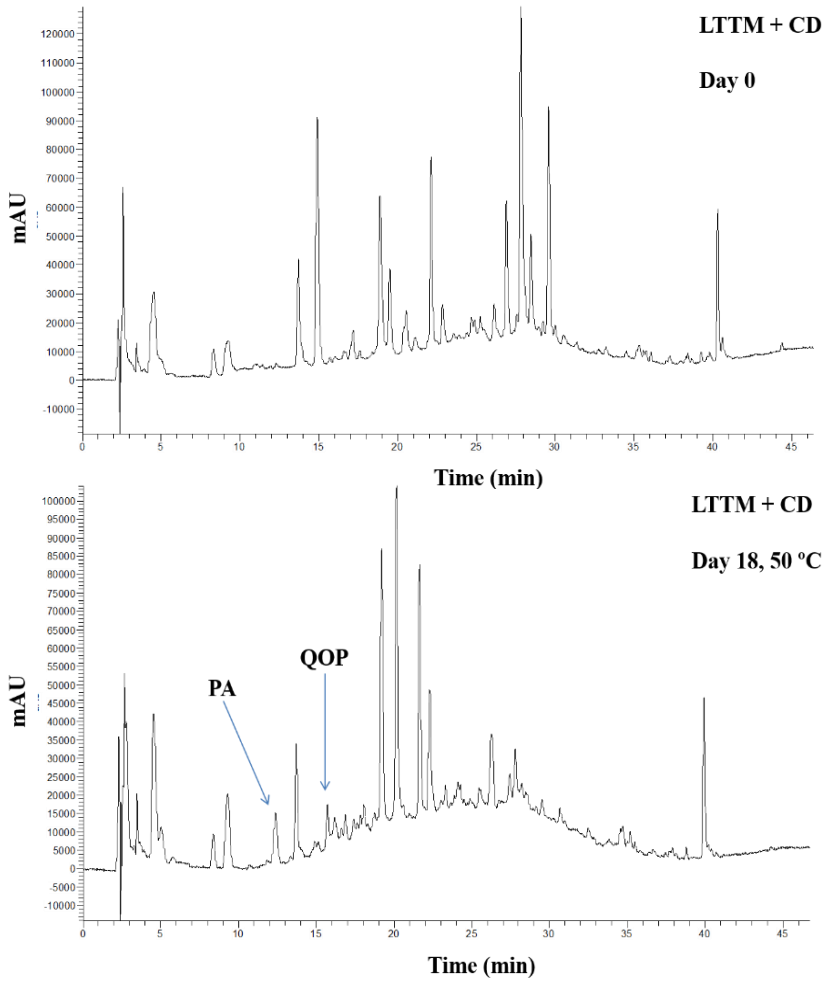

Fig. 3. Chromatogram of MoL extracts before (day 0) and after storage at 50 °C (day 18). The chromatogram was monitored at 280 nm. Peak assignment is as in the Table 2.

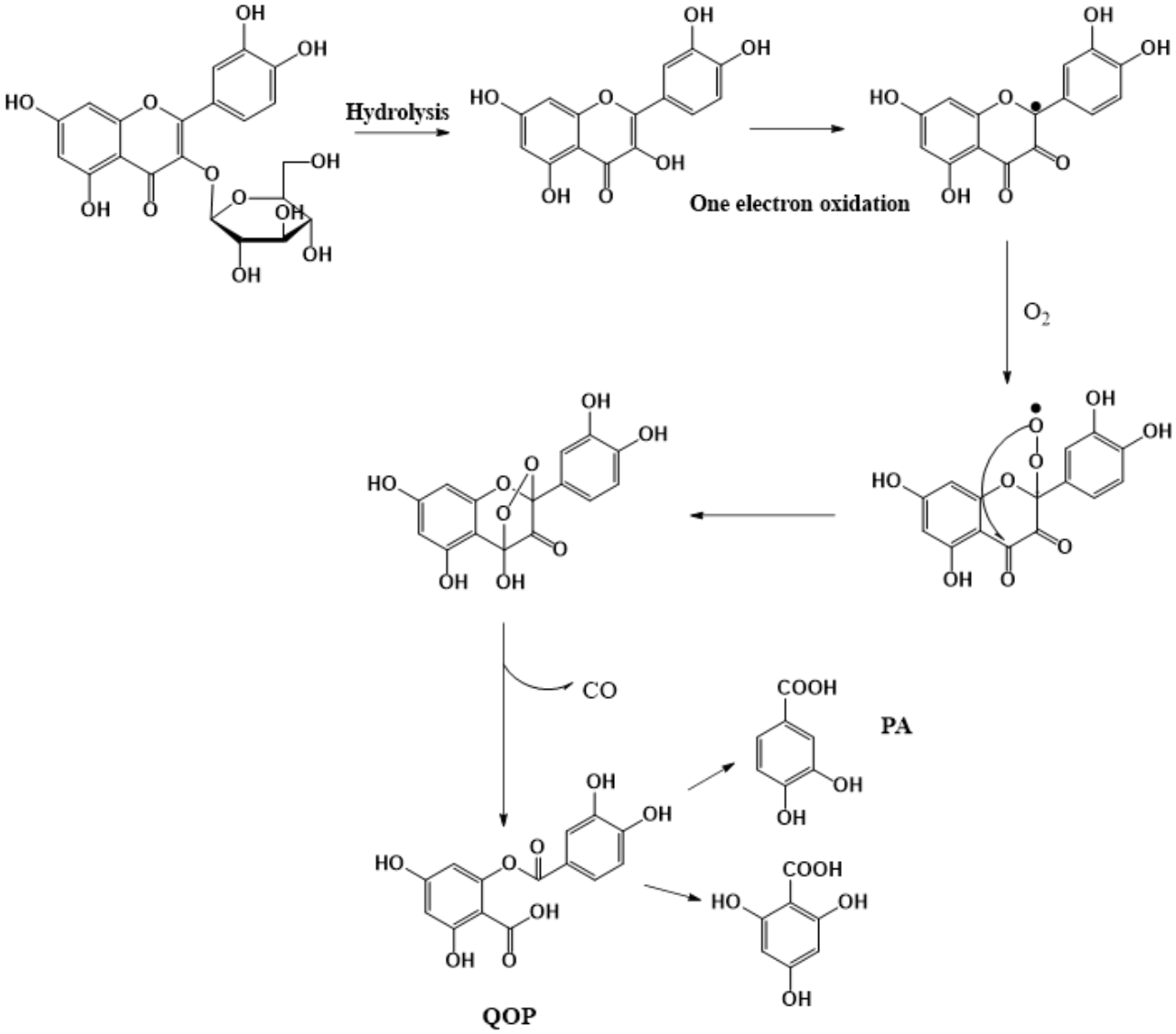

Fig. 4. Putative mechanism of quercetin glucoside degradation, resulting in the formation of PA and QOP.

After a cascade of reactions leading to the depside 2-(3,4-dihydroxybenzyloxy)-4,6-dihydroxybenzoic acid, PA may be formed through depside hydrolysis. The fact that extracts stored at 50 °C for 18 day displayed identical profile irrespective of the HP-*β*-CD presence (data not shown) indicated that HP-*β*-CD did not interfere with the oxidation mechanism, but it merely acted as protective agent retarding polyphenol degradation, as shown by the kinetic investigation. On the other hand, the clear evidence regarding flavonol degradation involving cleavage of the flavonoid skeleton was a strong background to justify A_{AR} decline of MoL extracts. This is because quercetin degradation products including PA and phloroglucinol carboxylic acid have been proven to be far less effective antioxidants than the parent molecule (Makris and Rossiter 2001). Therefore, oxidative decomposition of the quercetin glycosides in MoL extracts inevitably led to loss of A_{AR}.

Conclusions

LTTMs are novel solvents of unpredictable extraction behaviour and thus their characterisation in this regard is a matter of case experimentation. In the extraction of antioxidant polyphenols using LTTMs their stability is of high significance because polyphenols may undergo reactions, such as hydrolysis and oxidation. The polyphenolic profile of MoL extracts, obtained with either

LTTM/HP-*β*-CD or only LTTM, was demonstrated to be subject to modifications when stored for 18 days at 50 °C, characterised by flavonol glycoside degradation, which included cleavage of the flavonoid skeleton. Given that flavonoids were shown to be far superior antioxidants than their degradation products, the decline in A_{AR} seen in the stored MoL extracts could be attributed to the disappearance of these substances. However, the kinetic study showed that the incorporation of HP-*β*-CD in the extraction solvent may provoke significant decrease in A_{AR} decay. This outcome emphasized the importance of HP-*β*-CD for polyphenol stability in LTTMs, but additional examinations are needed to illustrate the mechanisms implicated. This is anticipated to lead in more effective HP-*β*-CD use in foods, pharmaceuticals and cosmetics.

Nomenclature

A_{AR}, antiradical activity [μmol DPPH g^{-1}; dw]
$A_{AR(0)}$, initial antiradical activity [μmol DPPH g^{-1}; dw]
$A_{AR(t)}$, antiradical activity at time t [μmol DPPH g^{-1}; dw]
k, pseudo first-order decay constant [$days^{-1}$]
t, time [days]
$t_{½}$, half-time [days]

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