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A one-pot ultrasound-assisted water extraction/cyclodextrin encapsulation of resveratol from *Polygonum cuspidatum*

Stefano Mantegna, Arianna Binello, Luisa Boffa, Marta Giorgis, Clara Cena, Giancarlo Cravotto*

Dipartimento di Scienza e Tecnologia del Farmaco, Università di Torino, Via P. Giuria 9, 10125 Torino, Italy.

*Author to whom correspondence should be addressed
(telephone +39 011.6707684; fax +39.011.6707687; *e-mail:* giancarlo.cravotto@unito.it)

Abstract

The ultrasound-assisted extraction (UAE) of resveratrol and other polyphenols from *Polygonum cuspidatum* has been carried out with the aim to developing an efficient and eco-friendly extraction process. The finely milled roots were sonicated (titanium horn, 19.5 kHz) in methanol, in different cyclodextrin water solutions [β-cyclodextrin (β-CD) or hydroxypropyl β-CD (HPβ-CD)] and also in pure water. UAE dramatically increased the yields and cut down extraction times compared to conventional extraction under stirring. Outstanding results have been achieved with the β-CD solution (1.5% w/w); in fact HPLC analysis showed that the selective inclusion properties of CDs toward phenolic stilbenes gave a much cleaner analytical extract profile. This green method gave 7.51 mg of total resveratrol (free + cleaved polydatin glucoside) per gram of dry plant. Thanks to polyphenol encapsulation within CDs, this extract showed excellent water dispersibility, higher stability and an antioxidant power which is comparable to that of the MeOH extract (DPPH, $ORAC_{FL}$). These important features should pave the road for its application in food supplements or phytochemical preparations.

Keywords: Resveratrol, polyphenols, *Polygonum cuspidatum*, ultrasound-assisted extraction, cyclodextrins

1. Introduction

Resveratrol and polydatin (piceid) are phenolic stilbenes synthesized by several plants as a defence response to situations of stress such as microbial infection, UV irradiation or mechanical damage (Okuda & Yokotsuka, 1996; Pezet, Perret, Jean-Denis, Tabacchi, Gindro & Viret, 2003). These molecules were found to be the major polyphenols present in the root of *Polygonum cuspidatum*. Their content is much higher in this plant than in grape berries. *P. cuspidatum* (Polygonaceae) is a perennial plant widely distributed in China, Japan and Korea and also found growing throughout North America. The dried roots of this plant were traditionally used in China and Japan as herbal folk remedies for the treatment of atherosclerosis, cough, asthma, hypertension and even cancer (Vastano, Chen, Zhu, Ho, Zhou & Rosen, 2000). Resveratrol and polydatin are widely used in medicine and health products on account of their important biological activities which include anti-

inflammatory, anticancer and cardioprotective action (Bertelli et al., 1999; Li, Chen, Huang, Lee & Su, 2000). The antioxidant properties of resveratrol and related compounds are certainly involved in these molecules' mechanism of action and because of the wide range of possible health benefits these polyphenols present, a huge effort has been made towards the development of isolation, purification and quantification methodologies. Conventional methods entail extraction by heating under reflux with ethanol, followed by filtration, concentration and purification. This procedure is time consuming and requires a large amount of solvent (Liu, Tang, Zhang, Bi, Wang & Zhao, 2007; Xiang, Zhou, Lei & Chen, 2005). Other authors have experimented with molecular imprinted polymers for the selective extraction and purification of resveratrol and piceid from the same plant (Zhuang, Dong, Ma & Zhang, 2008). Supercritical fluid extraction (SFE), which primarily uses CO_2 as its extraction medium, has been widely used for the extraction of non polar substances such as oils from natural plants and recent literature has shown that it can also be used for the extraction of resveratrol from *P. cuspidatum* when either ethanol or acetonitrile is added as a modifier (Yu, Shu & Zhao, 2005; Benova, Adam, Pavlikova & Fischer, 2010). Microwave-assisted extraction (MAE) has also been reported (Li, Li & Zhang, 2003; Wang, Dong & Xiu, 2008). The authors presented a series of advantages that their integrated method showed: short extraction time, reduced amount of solvent and direct concentration.

To the best of our knowledge ultrasound-assisted extraction (UAE) has never be applied to the extraction of this important natural source of resveratrol. It is well known that UAE technology can have a significant effect on the rate of various processes in the food chemistry (Chemat, e-Huma & Khan, 2011) and enhance the extraction of natural products from plant material, such as polyphenols, anthocyanins, flavonoids (Mason et al., 2011; Cravotto & Cintas, 2007). In a recent paper we have compared UAE, MAE and other extraction techniques to obtain phenolics from *Vitis vinifera* waste (Casazza, Aliakbarian, Mantegna, Cravotto & Perego, 2010). The shear forces created by the implosion of cavitation bubbles which are in turn created by ultrasonic waves disrupt plant tissues and facilitate extraction at room temperature. The easy scaling up of UAE, the safety aspects, the low energy consumption and the moderate investment required make this technique very attractive for industrial application. Sonication has been used to extract resveratrol and others phenolics from peanuts (Chukwumah, Walker, Verghese & Ogutu, 2009).

The conventional methods used to purify stilbene compounds from *P. cuspidatum* utilized a liquid-liquid partition together with silica gel column chromatography which required a large amount of organic solvents (Sun & Zhang, 2003). High-speed counter-current chromatography purification has recently been reported (Fan, Marston, Hay & Hostettmann, 2009), as has a two-step purification with macroporous resin adsorption and reverse-phase liquid chromatography (Zhang et al., 2009). All these protocols allow the recovery of resveratrol-enriched extracts although they are lacking in sustainability and are far from what would be expected by process intensification strategies.

Cyclodextrins (CDs) are well known for their ability to form inclusion complexes between bioactive compounds and their peculiar hydrophobic cavity (Cravotto, Binello, Baranelli, Carraro & Trotta, 2006) This increases the solubility, stability and bioavailability of the guest compound and therefore it is no surprise that their use in food and pharmaceutical industry is becoming ever more common (Szente & Szejtli, 2004). The relatively high hydrophobicity of resveratrol and its sensitivity to external agents such as air, light and oxidative enzymes constitutes a serious problem for its formulation, manipulation and bioavailability. An answer to these limitations is the inclusion of resveratrol in CD derivatives as has been suggested by several authors in a series of formulations (Lopez-Nicolas, Rodriguez-Bonilla & Garcia-Carmona, 2009; Lucas-Abellán, Fortea, Lopez-

Nicolas & Núñez-Delicado, 2007; Lu, Chen, Liu, Hu, Cheng, & Zou, 2008; Lu, Cheng, Hu, Zhang, & Zou, 2009).

In this piece of work we combine the UAE of *P. cuspidatum* roots and the CDs encapsulation of resveratrol and polydatin in one step. This is done using β-CD or HPβ-CD (1.5% w/w) in water. The strong favourable effect of power ultrasound on the extraction process has been demonstrated both in methanol and in CDs solutions.

2. Material and methods

2.1 Instrumentation

HPLC analyses were performed with a Waters 1525 Binary HPLC pump equipped with 2998 PDA and 2424 ELS detectors. UAE was performed using a high-power probe system with an immersion titanium horn, frequency 19.5 kHz in a power range of 50-100W. The extraction temperature was kept constant at 20°C by means of a thermostatted cooling bath.

UV analyses for DPPH tests were performed on Cary 50Bio spectrophotometer (Varian).

2.2 Chemicals and reagents

Analytical-grade methanol (Carlo-Erba Reagenti) was used for the preparation of samples and standards solutions of resveratrol and polydatin. The resveratrol (99%) and polydatin (>95%) reference samples were purchased from Sigma-Aldrich. β-CD and HPβ-CD were kindly provided by Wacher Chemie (Germany). HPLC-grade acetonitrile (Carlo-Erba Reagenti), Milli-Q water and formic acid (Sigma-Aldrich) were used as HPLC mobile phase.

2.3 Materials and sample preparation

For the calibration curve of resveratrol and polydatin, standard solutions at 1, 80, 160, 240, 320 and 480 µg/ml concentrations were prepared in methanol and a 30 µl amount of each was injected for HPLC analysis. Small portions (25 g) of *P. cuspidatum* roots were pulverized in a plant blender (Blendor, Warig Commercial) at a size range of 300-400 µm before extraction.

2.4 Extraction procedure

Finely milled roots (2 g) and solvent (50/100 ml of methanol or CD water solutions, see Table 1) were introduced into a three-necked pear-shaped 250 ml flask and the mixture was stirred at room temperature for 3 h or treated under US irradiation for 1 h at 20°C. US extraction started with a preliminary hard treatment of 5 min at 100 W for an effective cell wall disruption followed by a second step of 55 min at 50 W for an efficient mass transfer. Typical ratio solvent/plant was 50 ml/g. The suspension was centrifuged at 3500 rpm to obtain a clear orange-brown solution and the precipitate was washed with 10 ml of fresh solvent (methanol or water). The methanol extracts were concentrated under vacuum and then transferred to a 100 ml volumetric flask (final volume of 100.0 ml). 30 µl aliquots were used for HPLC analysis. The β-CD aqueous extracts were freeze dried overnight and 100 ml of methanol was added to precipitate β-CD and keep the phenolic compounds from the β-CD complex in solution. The white precipitate formed was washed with methanol (50 ml). The two methanol aliquots were collected, concentrated under vacuum and then transferred to a 100 ml volumetric flask (final volume of 100.0 ml). The same HPLC analysis followed.

2.5 HPLC analyses

The column used for analyses was a Luna C18 (5 µm, 250 mm x 4.6 mm; Phenomenex) and 0.4% formic acid in Milli-Q water (A) and acetonitrile (B) were used as the mobile phase. The gradient program started from 15% B up to 20% B over the 0-20 min period, from 20 to 40% B over 20-40 min, from 40 to 100% B over 40-60 min followed by a 100% B step over 60-65 min.

2.6 Determination of reactivity with DPPH•

The radical scavenging properties of the extracts were determined through reactivity with the stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) (Bondet, Brand-Williams & Berset, 1997). Ethanol solutions of the extracts (µg/ml) were prepared at different concentrations and rapidly mixed with an ethanol solution of DPPH• (final volume = 3 ml, final DPPH• concentration = 60 µM). The absorbance decay of DPPH• solutions after the addition of the extracts was followed for 30 min. In parallel, a blank solution of DPPH• was screened to estimate DPPH• decomposition during the measurement time. Initial and final DPPH• concentrations were exactly determined by interpolating radical absorbance values at t = 0 and at t = 30 min with a DPPH• standard curve performed in the concentration range 10 - 90 µM. Percentages of residual DPPH• concentration at 30 min were calculated and correlated *vs* extract scavenger concentrations, giving an exponential decay curve which was analyzed by non linear regression, to obtain EC_{50} values.

2.7 ORAC Assay

The ORAC test was performed using a Perkin-Elmer 2030 Multilabel Reader with 96-well black plates. 75 mM potassium phosphate buffer (pH 7.4) was used as a blank and different Trolox solutions, ranging from 0.25 to 6 µm, were used as standards (Ou, Hampsch-Woodill & Prior, 2001). The sample solutions were prepared by dissolving the extracts in an ethanol/buffer mixture (1/1 v/v) and diluting them with phosphate buffer. To start the incubation, aliquots of fluorescein (3′,6′-dihydroxyspiro[isobenzofuran-1[3*H*],9′[9*H*]-xanthen]-3-one) solution (150 µl of a 48 nM solution in potassium phosphate buffer) were dispensed into all wells first this was followed by 20 µl of either buffer, standard or sample solutions added (all in duplicate). The plate was covered and incubated in the preheated (37°C) microplate reader for 10 min which included 3 min shaking. 30 µl of AAPH (2,2′-azobis(2-amidinopropane) dihydrochloride) solution (133 mM in phosphate buffer) were added and the reaction started when the plate was inserted into the reader at 37°C. All fluorescence measurements were expressed relative to the initial reading of the fluorescence signal and were repeated every minute for 35 min at the emission wavelength of 535 nm with excitation at 485 nm. The net area under the curve (AUC) was calculated by subtracting the AUC of the blank from the AUC of either the standard or of the sample. The Trolox equivalent molar concentrations were calculated using a linear regression equation between Trolox concentration and the net AUC. To compare the antioxidant activity of the extracts we decided to calculate the relative ORAC values as Trolox micromoles present in 1 g of dried extract.

3. Results and Discussion

Conventional extraction processes for natural antioxidants such as polyphenols are quite laborious, time-consuming, involve large amounts of solvents and may ultimately cause some degradation of the target molecules. Great improvements in the process intensification of the extraction of resveratrol and polydatin from *P. cuspidatum* can be achieved with UAE, and efficient sonication can cut down the extraction time and increase the yields. Table 1 reports the experimental

conditions and the yields of resveratrol and polydatin which are calculated by molecular weight ratio. In all cases the extraction temperature was kept constant at 20°C by means of a thermostatted cooling bath.

Table 1.

Chromatograms **a** and **b** (Fig. 1) show the HPLC profiles of the β-CD sol. extract and the methanol extract respectively. The first peak corresponds to polydatin (retention time 19 min) and is followed by the free resveratrol peak (34 min). The CD selective inclusion property toward phenolic stilbenes caused the differences between the two chromatograms, the profile of the β-CD sol. extract (**a**) is much cleaner.

Figure 1

The optimization and validation of HPLC methods for the quantification of resveratrol and other polyphenols in *P. cuspidatum* has been the object of previous studies (Qian, Leung, Lu & Leung, 2008). Comparing these extracts, we observed that β-CD sol. gave a higher amount of free resveratrol (2.35 *vs* 2.15 mg/g) and less polydatin (9.64 *vs* 11.84 mg/g). The sugar portion in polydatin probably reduces its affinity to the CD and favours the in-out equilibrium. The influence of sonication was more evident in the extraction with β-CD sol. than for methanol. UAE showed an increase in total resveratrol content in β-CD sol. extract over plain stirring of 34%, while this increase was halved in the methanol extract (17%). A little less resveratrol was recovered when the plant/solvent ratio was decreased from 1:50 to 1:25 (1.78 *vs* 2.35 mg/g). The same was found to be true for polydatin (7.51 *vs* 9.64 mg/g). Finally the extract obtained using 25 ml of 3% HPβ-CD water solution per g of plant under UAE did not achieve the yields of the β-CD (0.85 and 4.98 mg/g for resveratrol and polydatin, respectively). Two marvellous and totally distinct colours were observed: yellow for the extract with β-CD and intense red for HPβ-CD (Fig. 2).
As described in the literature (Lopez-Nicolas, Rodriguez-Bonilla & Garcia-Carmona, 2009, and references therein), the inclusion complex between β-CD and resveratrol increases its stability in solution, preserving it from air and light degradation (*cis-trans* conversion). Moreover β-CD decreases the aggregation of resveratrol molecules, increases the bioavailability and the antioxidant activity.

Figure 2

In order to compare the antioxidant properties of the methanol and β-CD solution extracts obtained by UAE with the 1:50 plant/solvent ratio, their radical scavenging activities were investigated through reaction with DPPH• (Hsu, Chan & Chang, 2007) and the $ORAC_{FL}$ assay (Ninfali, Gennari, Biagiotti, Cangi, Mattoli & Maidecchi, 2009). The same assays were performed on the reference compound resveratrol (Lee, Seo, Lee, Chung & Chi, 2004; Ninfali 2009). The β-CD extract was re-extracted with methanol prior to use to obtain free-β-CD extract.
DPPH• is a stable radical which has been widely accepted as a tool for estimating the free radical-scavenging activities of herbal extracts. The results which are expressed as EC_{50} (μg/ml) and refer to the extract concentration needed to scavenge 50% of the initial DPPH• concentration after 30 min incubation, are reported in Table 2. Analysis of the values shows that both extracts obtained via the UAE of *Polygonum c.* behave as good radical scavengers regardless of the extracting solvent, with EC_{50}s comparable to that of reference compound resveratrol, a result which highlights the antioxidant properties of both extracts.

Table 2

In Figure 3a we report the time-dependent decrease in DPPH absorbance at 517 nm in the presence of similar concentrations of either MeOH extract, or β-CD extract of *P. cuspidatum*, or resveratrol: the equilibria between DPPH and scavengers are reached in different times.

Figure 3

More significant results in terms of antioxidant capacity are provided by the $ORAC_{FL}$ test which is based on the inhibition of the peroxyl radical-induced oxidation of fluorescein initiated by the thermal decomposition of 2,2'-azobis(2-amidinopropane)dihydrochloride (AAPH). The $ORAC_{FL}$ test is the only method that combines the evaluation of both the degree of inhibition and the inhibition time in a single quantity (ORAC value). The ORAC value has been widely used for the standardization of herbal extract and food antioxidant activity. The ORAC values obtained for the two extracts and for resveratrol are reported in Table 2, the resveratrol value is comparable with data reported in literature. Fluorescence decay curves induced by AAPH in the presence of different concentrations of either the sonicated β-CD extract or of the reference compound resveratrol are reported in Fig. 3b. Again the ORAC value obtained for methanol and β-CD extracts are comparable, a result which confirms the UAE with β-CD method as an excellent alternative to organic solvent extraction of the pool of antioxidants from *P. cuspidatum.*

4. Conclusion

A new extraction method, which avoids the use of organic solvents, was efficiently applied to the extraction of resveratrol and polydatin from the roots of *P. cuspidatum*. The extract obtained with a β-cyclodextrin water solution maintains the same radical scavenging activity and antioxidant capacity as the methanol extract obtained under the same conditions (sonication, plant/solvent ratio, elimination of extraction solvent prior to antioxidant tests). Moreover β-CD, if present in the final extract, could be a useful tool in improving the solubility, stability and bioavailability of resveratrol as an ingredient in foods. In conclusion, the combination of UAE and the capability of cyclodextrins to form inclusion complexes allow the use of water as the extraction media. The freeze dried or spray dried extract powder is an excellent active component for food supplements or phytochemical preparations.

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Figures Captions

Figure 1. HPLC chromatograms of UAE: β-CD sol. extract (**a**) and methanol extract (**b**).

Figure 2. From left β-CD and HPβ-CD extracts.

Figure 3. a) Decrease in DPPH absorbance in the absence or in the presence of either the β-CD extract, or MeOH extract or resveratrol; b) relative fluorescence decay curves of fluorescein in the absence and in the presence of different concentrations of β-CD extract or resveratrol.

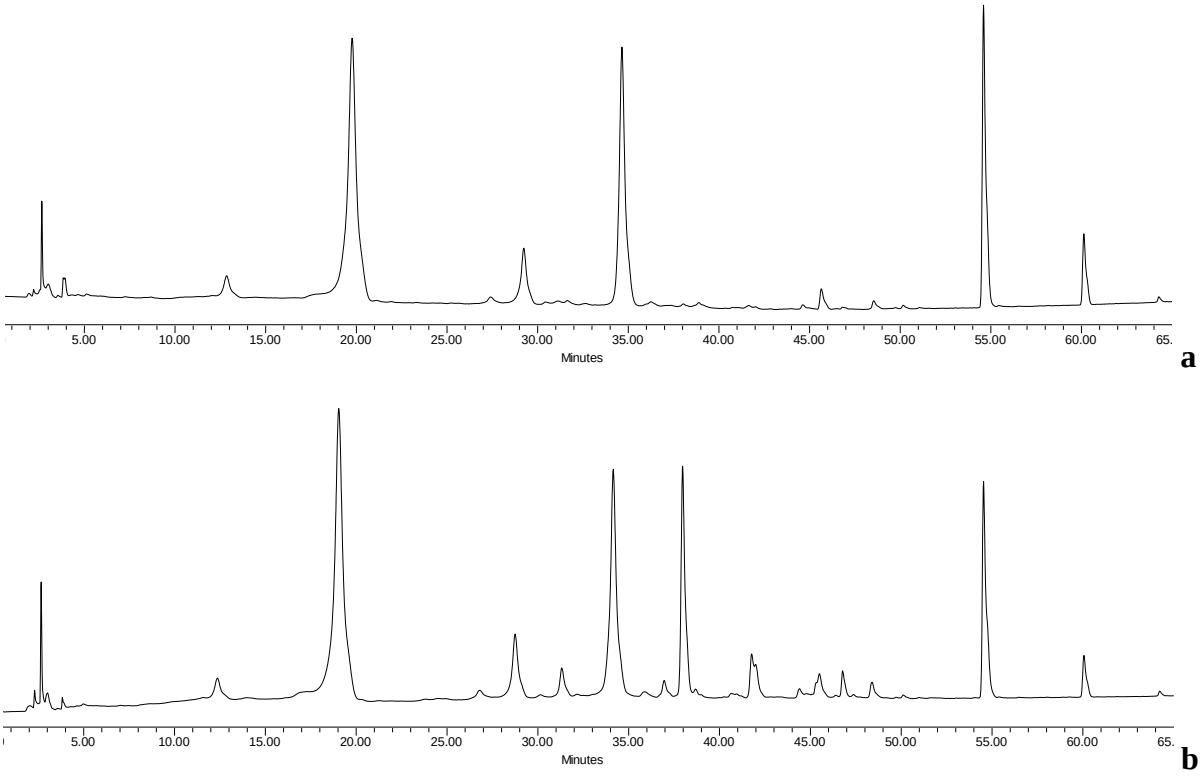
Table 1. Extraction conditions and yields (average of triplicate experiments).

Solvent (ml/g*)	**Method**	**Resveratrol "free"** (mg/g*)	**Polydatin** (mg/g*)	**Resveratrol "cleaved glucoside"** (mg/g*)	**Resveratrol "total"** (mg/g*)
Methanol (50 ml/g)	UAE#	2.15	11.84	6.92	9.07
β-CD sol. (50 ml/g)	UAE#	2.35	9.64	5.62	7.51
Methanol (50 ml/g)	stirring	1.89	9.62	5.64	7.99
β-CD sol. (50 ml/g)	stirring	1.48	6.49	3.79	5.27
β-CD sol. (25 ml/g)	UAE#	1.78	7.51	4.39	6.17
HPβ-CD sol. (25 ml/g)	UAE#	1.10	4.98	2.91	4.01
Water (50 ml/g)	UAE#	0.019	-	-	0.019

* Gram of plant. # UAE started with a preliminary hard treatment of 5 min at 100 W for an effective cell wall disruption followed by a second step of 55 min at 50 W for an efficient mass transfer.

Table 2: DPPH EC_{50} values and $ORAC_{FL}$ values for the two extracts obtained by UAE with methanol and β-CD solution with the 1:50 plant/solvent ratio and for resveratrol taken as reference compound.

	DPPH EC_{50} (µg/ml) (C.L. 95%)	**$ORAC_{FL}$± S.E.M.**
β-CD extract	18.4 (15.2 – 11.3)	9106 ± 909 (µmol TE/ g dry extract)
MeOH extract	8.3 (8.9 – 7.7)	7749 ± 617 (µmol TE/ g dry extract)
resveratrol	13.1 (11.3 – 15.2)	49642 ± 4315 (µmol TE/ g resveratrol)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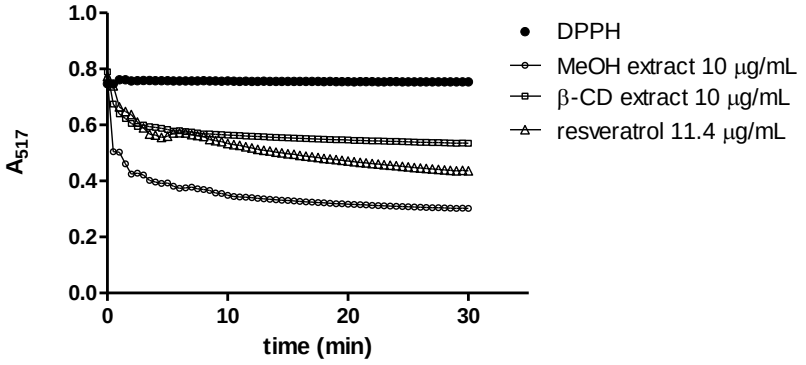
Minutes
a
b

Figure 1.

Figure 2

3a



3b

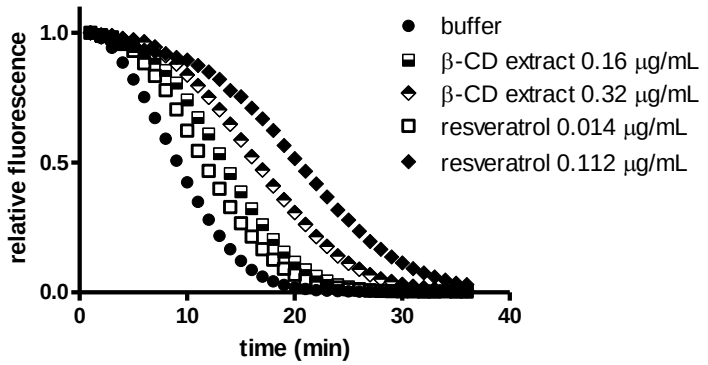


Figure 3