Journal of
Oceanography and Marine Research

Agatonovic-Kustrin, et al., Oceanography 2014, 2:4
DOI: 10.4172/2332-2632.1000e112

Editorial Open Access

Reversed Phase HPTLC-DPPH Free Radical Assay as a Screening Method for Antioxidant Activity in Marine Crude Extracts

Snezana Agatonovic-Kustrin[1*], David Mortona[1] and Pauzi A. Yusof[2]

[1]*School of Pharmacy and Applied Science, La Trobe Institute of Molecular Sciences, La Trobe University, Bendigo, Australia*

[2]*Physiology Department, Medical School, Universiti Teknologi Mara, Selangor, Malaysia*

[*]**Corresponding author:** Agatonovic KS. School of Pharmacy and Applied Science, La Trobe Institute of Molecular Sciences, La Trobe University, Bendigo, Australia, Tel: +61 3 5444 7360; E-mail: s.kustrin@latrobe.edu.au

Received date: Oct 28, 2014; **Accepted date:** Nov 25, 2014; **Published date:** Dec 3, 2014

Editorial

The large number of marine organisms present in the ocean, together with the diverse marine environments to which they have adapted, provides an enormous and mostly unexploited source of structurally novel and biologically active secondary metabolites [1]. Some of these organisms have provided compounds that have served as important leads in the discovery of new drugs [2], as probes in molecular pharmacology [3], and use as pest control agents [4]. In particular, they are a rich source of antioxidants but there has been limited work in screening organisms containing this rich source of structurally unique natural products for antioxidant activity. For instance, there are a large number of active components in macroalgae with antioxidant properties, including pigments such as polyphloroglucinols and fucoxanthin [5,6], bioactive carbohydrates such as fucoidan and laminarin, minerals, and UV absorbing mycosporine like amino acids [7]. Glutathione, an important antioxidant in plants, animals, fungi and some bacteria, is found in all macroalgae with some species containing as much as 3082 mg/100 g [8].

One way to screen crude marine extracts for antioxidant activity or radical-scavenging effects is to use either: (i) a solution based or (ii) a high performance thin layer chromatography (HPTLC) based 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical bioassay[9-12]. This method is based on the use of stable DPPH• free radical species which when added to a sample, react and neutralize antioxidants present in the sample [13,14]. DPPH• is a deep purple colored free radical that turns into pale yellow when reduced by an antioxidant. If a known amount of DPPH• is added to a sample, the decrease in absorbance at 517 nm due to the disappearance of DPPH• free radical is directly proportional to the amount of antioxidants present. The DPPH• free radical is stable in ethanol solution at room temperature as the odd electron on the nitrogen is able to delocalize through the entire molecule (Figure 1), so the molecules do not dimerise like most other free radicals. This delocalization of electrons gives rise to the intense violet color of DPPH solutions. It is the odd electron on the nitrogen atom in DPPH• that is reduced by receiving a hydrogen atom or electron from an antioxidant present to form its corresponding hydrazine (yellow non-radical species) [15]. The concentration of the DPPH at the end of a reaction will depend on the concentration and activity of the free radical scavengers presents [16].

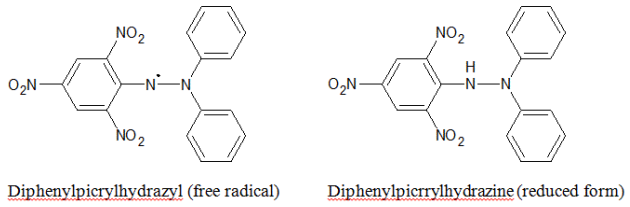

Figure 1: DPPH - free radical and its reduced form

In order to isolate and identify which are the most potent free radical scavengers present in a sample, the DPPH assay can be combined with either high performance liquid chromatography (HPLC) or high performance thin layer chromatography (HPTLC). Unfortunately, the use of the on-line HPLC method has not been successful as slow reaction kinetics results in inaccurate antioxidant capacity measurements [17]. HPTLC combined with DPPH radical detection of antioxidants *in situ* has been reported and successfully used to screen for antioxidants produced by marine bacteria and also antioxidants present in plant extracts [18]. In the HPTLC-DPPH assay, a developed plate with the separated sample components, is sprayed or dipped in an alcohol DPPH• solution. The yellow bands that develop on the HPTLC plate against a purple background indicate the presence of an active antioxidant compound.

Recently, we used a reversed phase HPTLC-DPPH• antioxidant assay using gallic acid as the model antioxidant analyte, to measure and compare the antioxidant activity of crude extracts in terms of their free radical-scavenging activity. The method performance on reversed phase plates is compared with the method performance on normal phase HPTLC plates. Normal phase and reversed phase HPTLC plates were spotted with the different plant extracts and then eluted. A suitable mobile phase for elution on reversed phase plates was developed using acetone: water with a final ratio of 9: 1 (v/v) providing good separation of the active compounds. In the case of normal phase HPTLC, best separation was obtained using n-hexane: ethyl acetate: acetic acid (20:10:1) as the mobile phase. Antioxidant activity was determined by spraying the developed plates with 0.4% (v/w) DPPH dissolved in ethanol. DPPH radical-scavenging compounds appeared as yellow bands against a purple background on the plate (Figure 2).

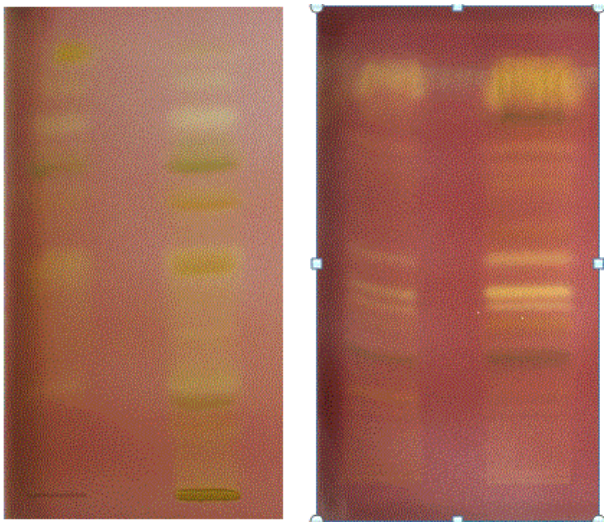

Figure 2: Developed reverse phase (left) and normal phase (right) HPTLC plate with white light under the plate after spraying with DPPH• solution.

The accuracy, precision, limit of detection (LOD) and limit of quantification (LOQ) for the quantification of gallic acid as a model analyte were determined using standard solutions of gallic acid. A linearity was established in the range of 0.5 -5.0 µg of applied gallic acid, with coefficient of correlations being 0.991 and 0.996 for normal and reversed phase HPTLC respectively. However, in the case of normal phase HPTLC method, the intercept was significantly different from zero (t=8.58, greater than a theoretical value t=2.36 at α=0.05) indicating method bias (Table 1). Higher precision of the method was also achieved using reversed phase TLC plates (Table 2). The accuracy of the normal phase HPTLC method was good except at the lower value of concentration studied (applied 1.0 µL) since it was below the LOQ.

Method	Equation of the line	Correlation coefficient	tcal	ttab	LOD (µg/µL)	LOQ (µg/µL)
Normal phase HPTLC	y=23445*x -17710	0.991	8.58	2.36	0.24	0.83
Reverse phase HPTLC	y=41104*x - 4944	0.997	1.84	2.13	0.33	1.11

Table 1: Calibration and regression of Gallic acid. y=Band area (pixels). x=Applied amounts (µL), tcal=calculated t value, ttab=tabular t value (α=0.05)

The hydrophobic layer in reversed phase HPTLC plates offers several advantages. Sample separation does not require the use of a complex multicomponent mobile phase. Acetone: water mixture can be used as a mobile phase and adequate separation can be achieved by modifying the fraction of water used. Plates are not affected by humidity and separation is better when compared with separation on normal phase HPTLC plates since the migration of the sample is based on partitioning rather than absorption. Reversed phase HPTLC plates are also generally recommended as they do not influence the radical antioxidant reaction.

However, reaction of positively charged silica gel present on normal phase HPTLC plates with the target polyphenolic compounds strengthens the DPPH radical antioxidant reaction and provides better color contrast on the plates [19].

	Applied volume (µL)	Observed volume (µL)	Relative standard deviation (%)	Bias (%)
Reverse phase HPTLC	1.0	0.98 ± 0.05	4.85	1.83
	2.0	1.94 ± 0.09	4.51	3.11
	3.0	2.94 ± 0.06	2.16	1.91
Normal phase HPTLC	1.0	1.06 ± 0.06	6.12	5.78
	3.0	3.00 ± 0.16	5.26	0.11

Table 2: Accuracy and precision of the method based on gallic acid quantification (n=3), Bias (%)=(observed volume- applied volume)/ applied volume.

References

1. Capon RJ (2001) Marine Bioprospecting - Trawling for Treasure and Pleasure. Eur J Org Chem 2001: 633-645.
2. Indap MM (2009) Drugs from the sea. Indian J Mar Sci 38: 7-9.
3. Coxon A, Bestor TH (1995) Proteins that glow in green and blue. Chem Biol 2: 119-121.
4. Peng J, Shen X, El Sayed KA, Dunbar DC, Perry TL, et al. (2003) Marine natural products as prototype agrochemical agents. J Agric Food Chem 51: 2246-2252.
5. Yan X, Chuda Y, Suzuki M, Nagata T (1999) Fucoxanthin as the major antioxidant in Hijikia fusiformis, a common edible seaweed. Biosci Biotechnol Biochem 63: 605-607.
6. Shiratori K, Ohgami K, Ilieva I, Jin XH, Koyama Y, et al. (2005) Effects of fucoxanthin on lipopolysaccharide-induced inflammation in vitro and in vivo. Exp Eye Res 81: 422-428.
7. Dunlap WC, Shick JM, Yamamoto Y (1999) Sunscreens, oxidative stress and antioxidant functions in marine organisms of the Great Barrier Reef. Redox Rep 4: 301-306.
8. Kakinuma M, Park CS, Amano H (2001) Distribution of free L-cysteine and glutathione in seaweeds. Fisheries Sci 67: 194-196.
9. Cuendet M, Hostettmann K, Potterat O, Dyatmiko W (1997) Iridoid Glucosides with Free Radical Scavenging Properties from Fagraea blumei. Helv Chim Acta 80: 1144-1152.
10. Brand WW, Cuvelier ME, Berset C (1995) Use of a free radical method to evaluate antioxidant activity. LWT - Food Sci Technol 28: 25-30.
11. Westenburg HE, Lee KJ, Lee SK, Fong HH, van Breemen RB, et al. (2000) Activity-guided isolation of antioxidative constituents of Cotinus coggygria. J Nat Prod 63: 1696-1698.
12. Ohtani II, Gotoh N, Tanaka J, Higa T, Gyamfi MA, et al. (2000) Thonningianins A and B, new antioxidants from the African medicinal herb Thonningia sanguinea. J Nat Prod 63: 676-679.
13. Wozniak D, Janda B, Kapusta I, Oleszek W, Matkowski A (2010) Antimutagenic and anti-oxidant activities of isoflavonoids from Belamcanda chinensis (L.) DC. Mutat Res 696: 148-153.
14. Huang D, Ou B, Prior RL (2005) The chemistry behind antioxidant capacity assays. J Agric Food Chem 53: 1841-1856.
15. Szabo MR, Iditoiu C, Chambre D, Lupea AX (2007) Improved DPPH determination for antioxidant activity spectrophotometric assay. Chem Pap 61: 214-216.

16. Naik GH, Priyadarsini KI, Satav JG, Banavalikar MM, Sohoni DP, et al. (2003) Comparative antioxidant activity of individual herbal components used in Ayurvedic medicine. Phytochemistry 63: 97-104.

17. Zhao J, Zhang JS, Yang B, Lv GP, Li SP (2010) Free radical scavenging activity and characterization of sesquiterpenoids in four species of Curcuma using a TLC bioautography assay and GC-MS analysis. Molecules 15: 7547-7557.

18. Takamatsu S, Hodges TW, Rajbhandari I, Gerwick WH, Hamann MT, et al. (2003) Marine natural products as novel antioxidant prototypes. J Nat Prod 66: 605-608.

19. Poole C (2015) Instrumental Thin-Layer Chromatography. (1st edn) Elsevier, Amserdam, Netherlands.