Spectrophotometric determination of total phenol in fish muscle after in- situ derivatization and reverse phase dispersive liquid- liquid microextraction

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Abstract

Reverse phase dispersive liquid- liquid microextraction (RP- DLLME) coupled with spectrophotometry is introduced for the detection of total phenol in the muscle tissue of fishes, such as *Sphyræna geniv*, *Otolithes ruber*, *Rastrelliger kangurta*, *Lutjanus johnii* and *Barbus Subquin Cunciat*. Phenols in fish tissues were extracted by ultrasonic bath and derivatization with 4- aminoantipyrine. The factors influencing on the extraction efficiency, including type and volume of extracting solvent, extraction time, concentration of 4- aminoantipyrine, derivatization reaction temperature and centrifugation rate and time were evaluated. The used protocol was found to yield a linear calibration curve in the concentration range of 0.4 and 1.6 mg L⁻¹ with a limit of detection of 0.012 mg L⁻¹. The enrichment factors founded 83 -fold. The SPSS was utilized for statistical analysis. The technique has been successfully used for the analysis of total phenols in fish species from several locations in Chabahar Bay.

Keywords: Reverse phase dispersive liquid- liquid microextraction, spectrophotometry, fish muscle, 4- aminoantipyrine

1. Introduction

Phenolic compounds are one of the most important pollutants of the environment. They are used in different manufacturing processes for products, such as plastics, dyes, synthetic rubber and household detergents (Khezeli & Daneshfar, 2015; Zhong *et al.* 2011). Because of their toxicity and carcinogenicity, many phenolic compounds are included in the list of priority pollutants in many

countries and monitored. For example, the European Community (EC) determines a legal tolerance level of 0.1 µg/L of each phenolic compound and 0.5 µg/L of total phenolics pollutants. So, establishing a simple, rapid and environment friendly technique for determining of such pollutants in environment and fish tissues is very crucial (Hashemi & Najari, 2018). For the detection of phenolic analytes different analytical technique, such as micellar electrokinetic chromatography (Ghorbanpour *et al.* 2014), capillary electrochromatography (Ghorbanpour *et al.* 2014), capillary electrophoresis coupled by ultraviolet (UV)

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detection or mass spectrometry (Feng *et al.* 2009; Blanco *et al.* 2005), high performance liquid chromatography (HPLC) (Hashemi & Najari, 2018; Feng *et al.* 2009) and spectrophotometry (Nassiri *et al.* 2014) has been proposed.

In analytical methods, sample pretreatment is generally needed for detection of trace analytes in real sample. Up to now, different extraction methods have been used to extract phenolic analytes from real samples, including ionic liquid dispersive liquid-liquid microextraction (Jiang *et al.* 2011), activated carbon (Li *et al.* 2009), air- assisted liquid- liquid solid phase extraction (SPE) (Masque *et al.* 2000), ion- exchange (Akhtar *et al.* 2006), dispersive liquid-liquid microextraction (Yang *et al.* 2017), ionic liquid three- phase liquid- liquid- liquid solvent microextraction (Guo *et al.* 2011), solid – phase microextraction (SPME) (Peñalver *et al.* 2002) and molecularly imprinted stir bar sorptive extraction (Hashemi & Najari, 2018). Rezaee and co-workers have introduced a novel microextraction method, referred to as dispersive liquid- liquid microextraction (DLLME) (Rezaee *et al.* 2006). The advantages of this technique include low cost, short extraction time and high recovery. This method has been used for microextraction of organophosphorus pesticides, phenols and thiamine in aqueous samples (Jiang *et al.* 2011; Berijani *et al.* 2006; Mohsen *et al.* 2010). DLLME is based on a ternary component solvent system. In this technique, usually, a small volume of organic solvent is dispersed into fine droplets in the sample solution with aid of a water miscible solvent as disperser. After phase separation achieved with centrifugation, the organic solvent is solidified at the conical bottom of centrifuge tube as a micro- drop which can be withdrawn using a microsyringe (Nassiri *et al.* 2018; Hashemi *et al.* 2017).

In this research, a new generation of DLLME, named as reversed phase DLLME was applied for pre- concentration and extraction of total phenols. In this protocol, a water phase is dispersed in organic solvent using disperser solvent. Thus, the sedimented phase which is a water microliter can be subjected to

spectrophotometry directly for determination of total phenols (Ziyaadini *et al.* 2016). The objective of this research is to develop the potential application of RP- DLLME for the microextraction of total phenols in fish muscle followed by determination with spectrophotometry for the first time in Chabahar Maritime University Bay research facilities.

2. Materials and Methods

2.1. Reagents

Phenol was purchased of Sigma- Aldrich (St. Louis, Mo, USA). All other salts, acids and organic solvents used as analytical grade and were obtained of Merck KGaA (Germany) and applied as received. Milli- Q[®] water (18.3 MΩ/ cm) was utilized throughout the runs. Stock solution of phenol (with concentration of 1000 mg/L) was prepared with dissolving the proper amount of phenol in n- hexane- dichloromethane (50:50). Concentration of total phenol in real sample was calculated with standard increase method. For determination of total phenol in fish muscle, the concentration of 0.5, 1.0, 1.5, 2.0 mg/L of the analyte in optimum amount of 4- AAP was separately added to 1 g of dried fish muscle that dissolved in 4 mL of n- hexane- dichloromethane (50:50 v/v). Then, concentrations of phenol in fish muscle are determined.

The SPSS (version 22) were utilized for statistical investigation. All data analyzed for normal distribution (Shapiro- Wilk) and two- way analysis of variance (ANOVA) applying SPSS software (at 95 % confidence level).

2.2. Apparatus

A UNICO S2100 Vis spectrophotometer (china) at wavelength of 510 nm coupled by 100 µL quartz microcells (model Q-01701, Stara company, UKZ) was applied for spectrophotometric detection of phenol. Model 340 i WTW (Germany) pH meter was used for detection of pHs. In Figure 1, the absorption

spectra of 1000 µg/L standard of phenol against blank reagent.

Fig 1: Absorption spectra for derivatization phenol by 4- AAP

2.3. Ultrasonic Extraction

The extraction of phenol of fish muscle was performed using the standard of EPA 3550C. In this method, 1 g of the dried fish muscle sample was spiked to 5.0 mL of dichloromethane- n- hexane (50: 50, v/v). With sonication for 15 min applying an ultrasonic bath, the mixture was shaken, then centrifuged for 15 min at 3500 rpm, the supernatant decanted into 15 mL conical glass sample tube and preserved for the next step.

2.4. RP- DLLME and Derivatization Protocol

Fifty mL of standard solution including, 1000 µg/L of phenol in n- hexane- dichloromethane (50:50 v/v) or 50 mL of real sample solution, obtained from ultrasonic extraction phase was placed in the test tube. An aliquot of 300 µL of acetone as dispersive solvent and 50 µL of 0.7 mol/L NaOH (the extraction solvent) was added and rapidly introduced to the diluted sample to form a cloudy solution. Next, the mixture was centrifuged at 4000 rpm for 5 min. The sedimented phase was removed with a 100 µL microsyringe and mixed with 50 µL of 0.9 w/v 4- AAP and 50 µL of 1.4 % w/v potassium

peroxodisulfate, adjusting pH to 9.5 with addition of 0.1 mol/L HCl solution. The solution was shaken in a 30 °C water bath for 10 min until the reddish- brown color formed. Finally, 100 µL of the water phase (extraction solvent) for determination of total phenols was introduced to UV-Vis spectrophotometry and read at wavelength of 510.

3. Results and discussion

3.1. Optimization of RP- DLLME for Phenol Extraction

To investigate the RP- DLLME pre-concentration operating parameters for phenols analysis, factors which could affect the RP- DLLME enrichment were investigated in 1000 µg/L of phenol.

3.2. Optimization of derivatization reaction

Derivatization of phenols using 4- AAP for UV- Vis detection was introduced by Emerson (1943). This technique is based on the oxidative coupling of phenols using 4- AAP in the presence of an oxidant to form antipyrine dyes, the concentration of that can be readily determined with UV-Vis. In the research, derivatization reaction is performed before the RP- DLLME protocol (Figure 2a). The use of colorimetric techniques has been reported for following the reaction of phenol by 4- AAP, either unsubstituted or ortho- or meta- substituted using halogens or methoxy, carboxy or sulfonate groups as seen in Figure 2b. Production of dyes is carried out in the presence of oxidants and our research indicated which potassium peroxodisulfate is superior to other suggested reagents (Nassiri et al. 2014; Ziyaadini et al. 2016). The reaction at the pH of 9.5 leads to the generation of stable reddish- brown colored antipyrine dyes readable by spectrophotometry in water solution at wavelength of 510 nm (Nassiri et al. 2014; Ziyaadini et al. 2016).

3.3. Effect of 4- Aminoantipyrine (4- AAP) Concentration

The concentration effect of 4- AAP on dye production was studied in range 0.01- 0.1 mol/L. It was found that the production of complex is quantitative for concentration more than 0.05 mol/L of 4-AAP, further runs performed with saturation of the samples by 4- AAP.

3.4. Effect of Derivatization Reaction Temperature

The effect of temperature of derivatization reaction was studied. Absorption of phenols from organic phase significantly increased with increasing the parameter up to 27 °C and became stationary after that.

Fig 2.a: Reaction scheme for phenol treated with 4- AAP, b. production scheme of dyes in the presence of proper of oxidants a standard of phenols in organic media.

3.5. Effect of Concentration of Extraction Solvent

Phenolic analytes are present in hydrophilic salt in the alkaline condition (Liu et al. 2013). So, phenolic compounds can be transformed from organic phase to their alkaline aqueous phase. 50 μ L of sodium hydroxide using various concentrations (0.1- 1.0 M) was utilized for analysis the spiked blank sample. The results are given in Figure 3. By increasing the extraction solvent volume from 0.1 to 1.0 M, the

absorbance intensity hiked, but when the concentrations of NaOH exceeded beyond 0.7 to 1.0 M, the absorbance intensity decreased. This is because optimum pH changed from that proposed for RP-DLLME technique with increase in the concentration of NaOH (the reason for the sharpness of the curve after the optimal value). Higher extraction efficiencies were achieved by a 0.7 M NaOH and then decrease occurred (Figure 3).

Fig 3: Effect of concentration of NaOH as extraction solvent on absorption intensity

3.6. Effect of Concentration of Extraction Solvent

Different volumes of 0.7 M NaOH solution between 30 and 80 μ L in 5 μ L intervals were subjected to RP-DLLME. When the volume of the extraction solvent was increased from 30 to 50 μ L, the increase in the absorbance was not remarkable, however, with the volume over 50 μ L, no distinct changes on the absorbance occurred., thus in the subsequent experiment a volume.

3.7. Effect of Disperser Solvent

The water as extraction solvent is dispersed as

very fine droplets in the organic phase using a moderately polar disperser. The dispersed solvent should be miscible by the solvent extraction and the extraction solvent and the organic phase. Therefore, ethanol, acetonitrile, acetone and ethyl acetate as disperser solvents were tested. Ethanol distinguished the quantitative extraction of total phenol from organic phase (Figure 4).

This is probably because of ethanol is a polar aprotic solvent with relatively high dielectric constant. Also, the disperser solvent has low surface tension and high surface activity.

3.8. Effect of Volume of Disperser Solvent

The volumes of disperser solvent were investigated to improve the recoveries of total phenols. The increase of the disperser volume (100-800 μL) slightly improved RP- DLLME efficiency of

analytes to 300 μL , but above this volume, the absorption remains constant as shown in Figure 5 (based on experimental result). Based on the results of the DLLME optimization study, 300 was selected as optimal volume of disperser solvent.

Fig 4: Effect of type of disperser solvent on absorption intensity

Fig 5: Effect of disperser solvent volume on absorption intensity

3.9. Effect of Extraction Time

The time factor was investigated in the range of 1-10 min. The extraction efficiency reached its maximum value at 3 min and remained constant afterwards. Thus, 3 min was selected to all runs.

3.10. Effect of Centrifugation Rate and Time

The effect of centrifugation rate and time on the extraction efficiency was investigated in the range of 1000- 4000 rpm and 1-10 min, respectively. A centrifugation time of 2 min and 2000 rpm as centrifugation rate were selected for the further runs as complete phase separation occurred and became constant after that.

3.11. Analytical Performance

3.11.1. Linearity, limit of detection and enrichment factor

Under optimum condition, the calibration curve was linear over a concentration range of 0.02- 5 mg L⁻¹ for phenol. The least square equation above the dynamic linear range is

$$A = 0.1232 C + 0.0222 \text{ with } r^2 = 0.9896$$

Where C and A are the concentration of total phenol response.

The enrichment factors (EFs) and recoveries were achieved with Eqs. (1) and (2), respectively (Hashemi et al. 2017).

$$EF = C_{org} / C_{aq} \quad (1)$$

$$R = (C_{org} \times V_{org}) / (C_{aq} \times V_{aq}) \times 100\% = EF \times (V_{org} / V_{aq}) \times 100\% \quad (2)$$

Where EF, R, C_{aq}, C_{org}, V_{org} and V_{aq} is the enrichment factor, recovery, initial concentration of analyte in the sample, sediment phase (extraction solvent volume) and water sample volume, respectively.

Under optimal experimental parameter, the limit of detection (LOD) of the investigated protocol for phenol was calculated 12 µg/ Kg for the analyte based on the 3 Sd/m criteria; in which Sd is the standard deviation of seven consecutive measurements of the blank and m is the slope of calibration curve. The enrichment factors (defined as Eqs. 1) founded 83 -fold. Table 1 compares the characteristic data of the proposed technique with those utilizing solid phase extraction for phenol compounds determination, reported in the literature.

3.11.2. Determination of Phenols in Fish Muscle

The studied area was selected in Chabahar Bay in this research based on the high probability of phenol contamination in the area. Fish specimens were collected in winter 2018 from all the sampling stations and then, placed in a plastic bag in the ice container and transferred to the laboratory and stored in the freezer at -20 ° C until analysis. During the analysis, the tissues were separated and chopped with a meat grinder. Prior to analysis, chopped fish samples were kept at -40 ° C for 48 hours in a freeze dryer. After drying, they were milled again and then separated into particles less than 0.2 mm mesh size and prepared for subsequent steps.

To investigate the performance of the investigated technique, detection and determination of phenol in fish muscle were tested. The results are shown in Table 2. These results show that the introduced procedure can be used for enrichment and determination of phenol in fish muscle.

Table 1: Comparison of the published techniques for determination of phenolic compounds with the suggested protocol in this work

Method	Detection method	LOD ($\mu\text{g L}^{-1}$)	Linear range ($\mu\text{g L}^{-1}$)	Ref.
SOPME	HPLC	0.27	0.27- 53.50	(Jiang <i>et al.</i> 2011)
IL- LLL- SBME	HPLC- UV	0.1	0.5- 50.0	(Guo & Lee, 2011)
SPME	HPLC- UV	2-4	NM	(Peñalver <i>et al.</i> 2002)
Capillary liquid chromatography	Electrochemical detection	1	3- 50	(Segovia-Martinez <i>et al.</i> 2010)
SPE	HPLC- UV	50- 1000	NM	(Saitoh <i>et al.</i> 2002)
RP- DLLME	UV- Vis	15 $\mu\text{g Kg}^{-1}$	50- 1800 $\mu\text{g Kg}^{-1}$	(Ziyaadini <i>et al.</i> 2016)
RP- DLLME	UV- Vis	12 $\mu\text{g Kg}^{-1}$	20- 5000 $\mu\text{g Kg}^{-1}$	This work

NM, Not mentioned; SOPME, salting out phase separation microextraction; IL- LLL- SBME, Ionic liquid three- phase liquid- liquid- liquid solvent bar microextraction.

Table 2: Determination of total phenols with proposed RP- DLLME in fish muscle of Chabahar Bay (Iran)

Type of fish	Percentage of dried fish	Fish size (cm)	Phenol founded ($\mu\text{g Kg}^{-1}$)
<i>Sphyræna genie</i>	75.6	37.8	0.063
<i>Otolithes ruber</i>	76.9	26.2	0.103
<i>Rastrelliger kangurta</i>	75.0	24.0	0.126
<i>Lutjanus johnii</i>	76.0	22.2	0.087
<i>Barbus Subquin (Cunciatus)</i>	75.0	37.8	0.148

The result showed the significant difference between concentrations of total phenols in *Barbat subquin* (Cunciatus) with *Rastrelliger kangurta* and *Lutjanus johnii* of fish samples. Also, content difference of total phenols in *R. kangurt* and *Sphyræna genie* is significant. Content of target analyte in *Barbut ubquin* (Cunciatus) with *Sphyræna genie* and *Lutjanus johnii* was significant difference.

4. Conclusions

In this paper, a novel RP- DLLME method was successfully developed and tested for pre-concentration and spectrophotometric determination

of total phenols in the tissues of five fish species from Chabahar Bay. The linear range had a wide concentration and the RP- DLLME could selectively extract phenols for spectrophotometry determination even at low concentration. RP- DLLME has many advantages. For example, it is easy to extract with simple operation, is repeatable, allows rapid separation, costs less and has good selectivity in complex sample with good linearity in results.

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