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FULL PAPER TAM MAKALE

A RAPID HPLC METHOD FOR DETERMINATION OF 4-HEXYLRESORCINOL RESIDUES IN SHRIMP

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Abstract: A rapid method for the extraction, separation and quantification of the 4-hexylresorcinol from shrimp with high performance liquid chromatography (HPLC) is described. This modified method is faster than previously described methods with short pre–preparation and sharp resolution in HPLC procedures (sample preparation, extraction, separation and quantification total 40 min.). The coefficients of determinations (r^2) measured for 4-hexylresorcinol were obtained over 0,999 in each curve. The confidence interval of the recovery working range of 1.5–2.5 mg/kg was approximately 95%. Limits of detection and quantitation were determined as 0.04 and 0.06 mg/kg for shrimp.

Keywords: 4-hexylresorcinol, Shrimp, Melanosis, Residue levels, HPLC

Öz: **Karideslerde 4-hexylresorcinol kalıntılarının belirlenmesi için hızlı bir HPLC Metodu**

Bu makalede, karidesten 4-hexylresorcinol'un ekstraksiyonu, yüksek basınçlı sıvı kromotografisinde (HPLC) ayırımı ve miktarının belirlenmesi ile ilgili hızlı bir metot anlatılmıştır. Bu modifiye metot, kısa ön hazırlık ve HPLC' de keskin pik ayırımı ile daha önceki metotlara gore daha hızlıdır (örnek hazırlığı, ekstraksiyon, ayırım ve miktarın belirlenmesi toplamda 40 dakika). İki eğride de korelasyon katsayısı (r^2) 0,999 un üzerinde ölçülmüştür. 1.5–2.5 mg/kg lık geri kazanım çalışma aralığının güvenilirlik değeri yaklaşık % 95 bulunmuştur. Tespit limiti ve ölçüm limiti 0.04 ve 0.06 mg/kg olarak bulunmuştur.

Anahtar Kelimeler: 4-hexylresorcinol, karides, Melonosis, kalıntı seviyesi, HPLC

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Introduction

Color change called "melanosis (black spots)" consists in shrimp and crustacean as a result of enzyme activity during storage (Figure 1.). Melanosis has no health risk for consumer. This color change is the main reason for economic loss in world shrimp trade which is accepted as spoiled product in the eyes of the consumer. For the solution of this trouble, many chemicals have been developed for commercial applications. One of them is 4-hexylresorcinol that its use has increased in recent years. 4-hexylresorcinol prevents from polyphenol oxidase (PO) that it is found under shrimp shell and in it. Thus, melanosis is delayed. But 4-hxylresorcinol remains residue such as other chemicals and its usage is limited with legal limit values (Collins-Williams 1983; McEvily et al. 1991; Montero et al. 2006, Mendes et al. 2006). Maximum acceptable residue levels for this agent in raw material in China and Canada is 1 mg/kg, whereas it is accepted as 2 mg/kg in European Union (GB 2760-1996, 1996; Food and Drug Regulation 1078, 1998; EU Scientific Committee on Food, 2003).

Figure 1. Melanosis formation in shrimp (Varlık et al., 2007)

4-hexylresorcinol is a new additive in fisheries because it inhibits development of enzymatic browning melanosis in a variety of storage situations which developes rapidly in shrimp, in lobsters after catching and during iced storage (Montero et al. 2004; Mendes et al. 2006).

The detection and quantification of 4-hexylresorcinol in shrimp are achieved by HPLC. Reverse phase HPLC method coupled with florescence detector is preferred for quantification of 4-hexylresorcinol levels in food, because of its better selectivity, accuracy and sensitivity.

The objective of this study was to investigate determination of 4-hexylresorcinol levels in shrimp flesh compared to other methods in terms of analysis time and sensitivity.

Materials and Methods

Samples

Deep water pink shrimp samples (*Parapenaeus longirostris*, Lucas 1846) were collected from the Marmara Sea, Turkey.

Determination of 4-hexylresorcinol Residues

4-hexylresorcinol value was analyzed by a HPLC method modified from Jonker and Dekker (2000).

Chemicals and laboratory equipments: 4-Hexylresorcinol (%99) (Acros Organics, Catalog No. 197920250, Belgium), Methanol (Merck gradient grade for liquid chromatography LiChrosolv® Reag. Catalog No. 1.06007.2500, Germany), Acetonitrile (Merck gradient grade for liquid chromatography LiChrosolv® Reag. Catalog No. 1.00030.2500, Germany), Potassium dihydrogen phosphate (Merck Catalog No. 1.04873.1000, Germany), Ortho – Phosphoric Acid (Merck Catalog No. 1.00573.1000, Germany), Deionized water, 0.22 µm, 13mm syringe membran type nylon filters (E-Chrom Tech, Taiwan), Volumetric flask (25 mL and 50 mL), Measuring cup (10 mL, 25 mL and 100 mL), Automatic pipette (10 µL, 100 µL and 1000 µL), Syringe (10 mL) and Centrifuge tube (50 mL)

Instrument: Shimadzu LC 10 AT Vp series pump, Shimadzu SIL 10AD Vp cooling automatic sampling (4°C), Shimadzu RF 10AXL fluorescence detector (FLD), Shimadzu CTO 10AV Vp, Shimadzu SCL 10A Vp and Shimadzu Class-Vp 6.14 (Shimadzu Corporation, Japan).

Chromatographic conditions:

Injection volume: 50 µL

Flow rate: 0.8 mL/min

Mobile phase: 40/60 (Phosphate buffer solution (0.01 M Phosphate buffer solution was prepared by dissolving 1.36g KH_2PO_4 in 1 L deionized water and was adjusted to pH 3 with %25 H_3PO_4)/Acetonitrile)

Detector: Fluorescence (Excitation wavelength: 280nm, Emission wavelength: 310nm)

Column: ACE C_{18} 250mm x 4.6 mm x 5µm (Advanced Chromatography Technologies Ltd, Scotland)

Column oven temperature: 35°C

Analysis time: 10 min

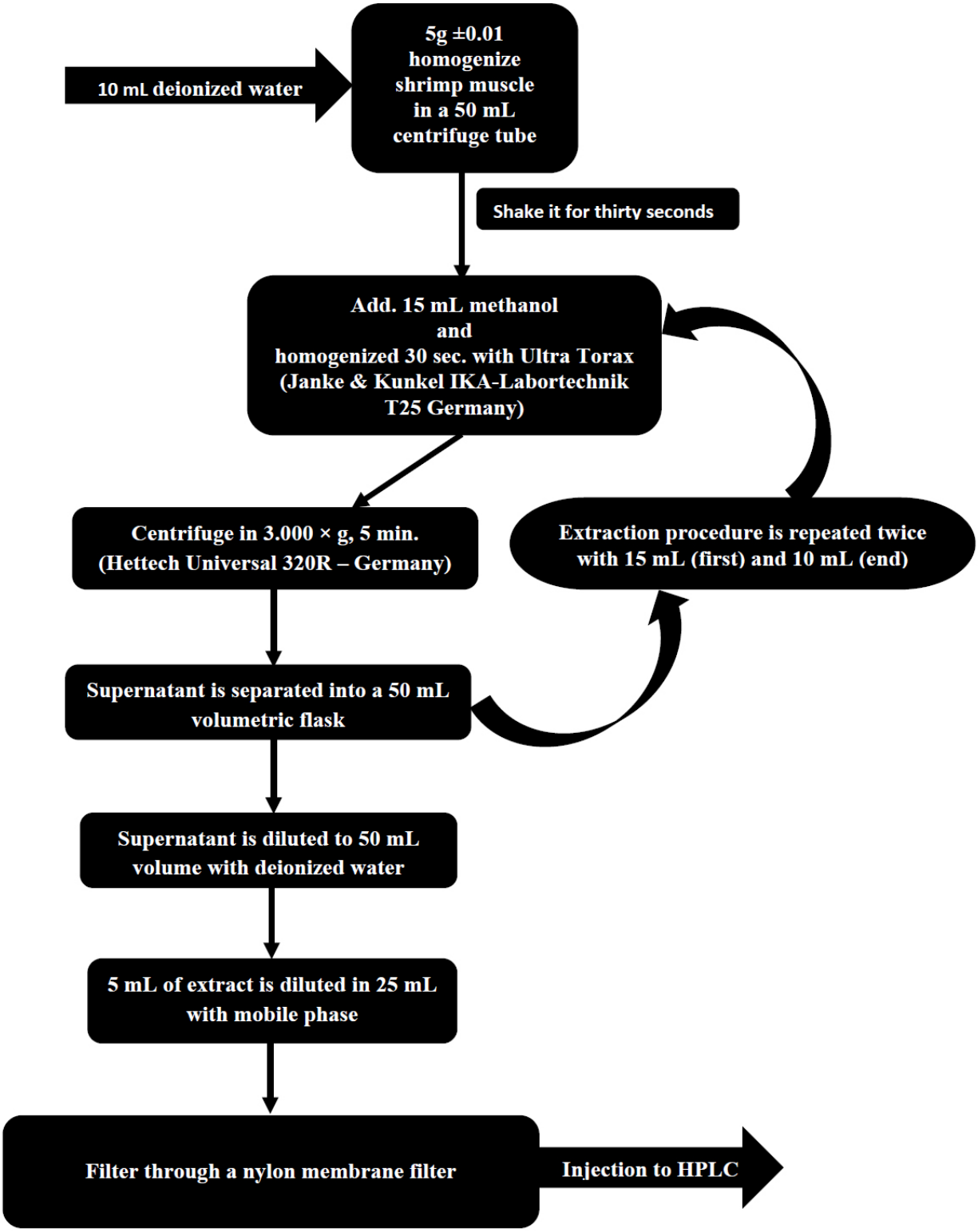
Standard solution: Stock standard solutions (500 ppm) of 4-hexylresorcinol was prepared by dissolving 50 mg in 100 mL of methanol. The standard solution can be stored for at least 6 months in brown glass (in freezer -18°C). Buffer stock solution (5 ppm) was prepared by dissolving 1 mL stock standard solution in 99 mL of methanol. Working standard solutions in the concentration of 1, 5, 10, 50, 100, 250, 500 and 1000 ppb were prepared daily by dilution of buffer stock solution in mobile phase.

Calculation: The concentration of 4-hexylresorcinol (µg 4-hexylresorcinol/L) was calculated from the regression equation derived from the standard curve.

4-hexylresorcinol was calculated from the equation:

$$4-\text{hexylresorcinol (mg/kg)} = \frac{HPLC\ value\ read\ (\mu g/L) \times 0.250\ L}{Sample\ weight\ (g)}$$

Analysis steps:



Method Performance Criteria

Limit of detection (LOD), Limit of quantification (LOQ), accuracy (recovery- trueness, repeatability and within reproducibility – precision) have been studied to determine method performance criteria by single laboratory validation.

LOD and LOQ: LOD and LOQ were determined by analyzing 10 independent fortified samples at a very low level and calculated with mean plus 3 standard deviation and 10 standard deviation, respectively.

Accuracy: For accuracy study, two levels spiked samples were prepared and 18 replicates have been performed in each level. 1 mL of 4-hexylresorcinol was added into blank samples from 7,5 mg/L and 12 mg/L standard solutions to obtain 1.5 and 2.5 mg/kg concentrations respectively. Samples have been stored in a dark and ambient temperatures for 2 h before extraction. For each level, 18 samples were analyzed as 6 replicates in three independent analytical runs. Repeatability and within reproducibility were calculated according to ISO 5725-2 (1994) expressed as coefficient of variation. Recovery was calculated according to Eurachem Guide and expressed as percentage.

Linearity: Two calibration curves were prepared at 5 concentration levels and triplicate measurements at each level (**Curve 1**: 1, 5, 10, 50 and 100 ppb, **Curve 2**: 100, 250, 500, 750 and 1000 ppb) by linear regration. 4-hexylresorcinol concentration in samples were determined with the aid of the instrument software by using the calibration curve.

Validation scheme: The present method was optimized and validated according to "The Fitness for Purpose of Analytical Methods – A Laboratory Guide to Method Validation and Related Topics" (Eurochem Guide-1998).

Statistical Analysis

Results were presented with mean and standard deviation of values (n=3) in the tables. The standard deviation and coefficient of determinations (r^2) were determined using STATISTICA 7 (StatSoft Tulsa, Oklahoma-USA).

Results and Discussion

4-hexylresorcinol in shrimp

Typical HPLC chromatograms of 4-hexylresorcinol were presented in Figure 3 and Figure 4. 4-hexylresorcinol was separated at 280 nm (excitation) and 310 nm (emission) wavelength in less than 10 min.

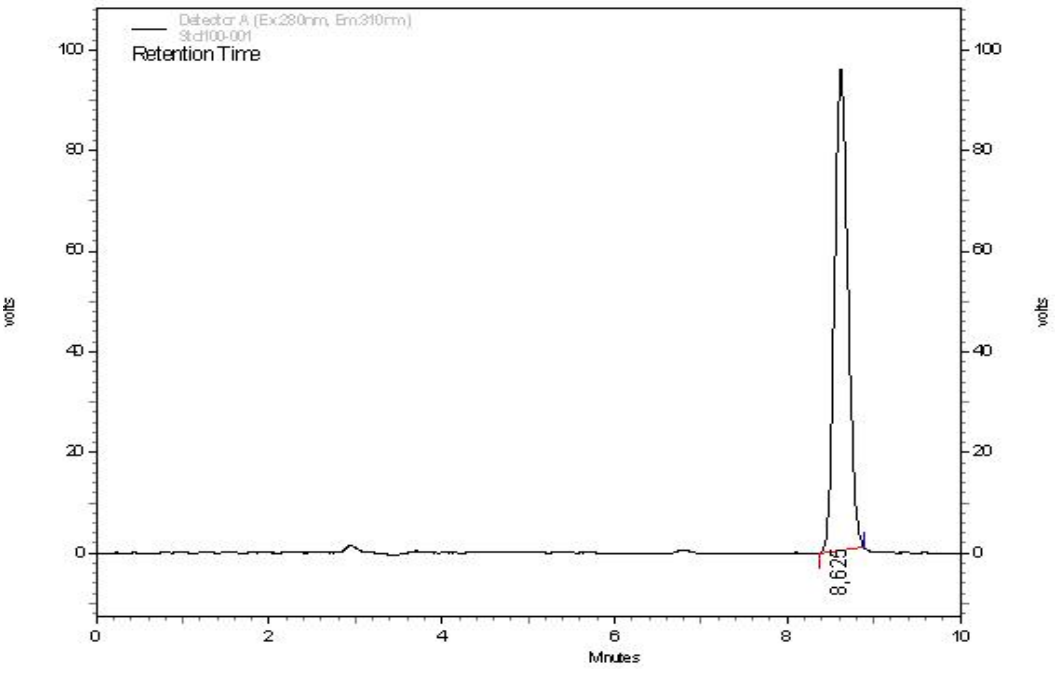


Figure 2. 4-hexylresorcinol standards peak

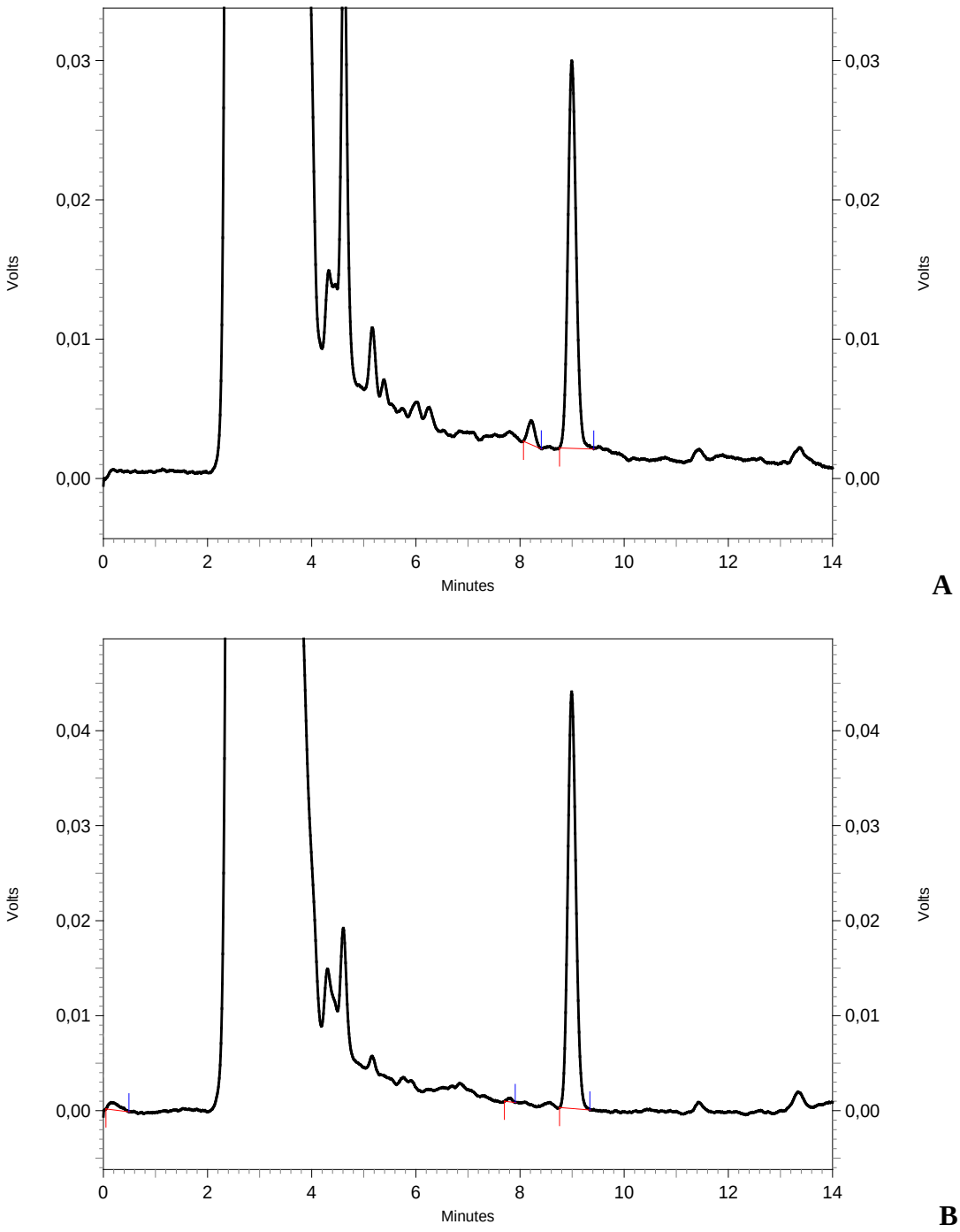


Figure 3. 4-hexylresorcinol spiked shrimps chromatograms at the levels of 1.5 (A) and 2.5 (B) mg/kg

LOD and LOQ

LOD and LOQ values of the method for 4-hexylresorcinol were presented in Table 1.

Table 1. LOD and LOQ of the method for 4-hexylresorcinol

LOD (mg/kg)	LOQ (mg/kg)
0.04	0.06

Accuracy

The recovery, repeatability and reproducibility ranged between 94.68 to 94.80%, 1.23 to 2.51% (CVr), and 1.89 to 2.74% (CVR), respectively (Table 2). General acceptance range is between %70-110 for recovery studies and recovery obtained in this study was good in trueness. These results show that the method has a good precision due to its high recovery and low CV_r and CV_R values.

Table 2. Results for recovery, repeatability (CV_r) and reproducibility (CV_R) in 4-hexylresorcinol

	N	1.5 (mg/kg)	2.5 (mg/kg)
Day1	6	1.41	2.37
Day2	6	1.45	2.41
Day3	6	1.42	2.34
Mean		1.43	2.37
SD (±)		0.04	0.04
CV_r (%)		2.51	1.23
CV_R (%)		2.74	1.89
Recovery (%)		94.68	94.80

Linearity

Regression analysis was performed for calibration and correlation coefficients (r^2) were obtained over 0,999 in each curve (Table 3).

Jonker and Dekker (2000) have found the pooled recovery 81.6%. The confidence interval was ranged from 81.6 % to 95 %. The relative standard deviation (RSD) for the range of application (1.5–2.5 mg/kg) was 2.1 %.

Table 3. Linearity of 4-hexylresorcinol

4-hexylresorcinol	Regression equation	r^2
Linearity 1	$9.69477x10^5$	0.999795
Linearity 2	$9.72366x10^5$	0.999106

With this work, we decreased the time for preliminary preparations and increased sensitivity of the study.

Conclusions

It can be concluded that the presented method (HPLC-Fluorescence) has potential to be used for quantification of 4-hexylresorcinol in shrimps due to its rapidness, simplicity, reliability and sensitivity. The validated method has a good overall recovery, repeatability and reproducibility and also low LOD and LOQ value. It can separate 4-hexylresorcinol at 280 nm (excitation) and 310 nm (emission) wavelength in less than 10 min and also provides minimal sample preparation. The average analysis time (sample preparation, extraction, separation and quantification) takes approximately 40 min.

Acknowledgements

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