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Research Article

Halal Gelatin Analysis Using Developed On-line RP-HPLC-DAD-UV Henna Protein Detection in Confectionery and Food Supplements

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Abstract

Isobutanolic infusion of henna plant, which inspired the perfume industry with its pleasant scent used in Islam, Judaism and Christianity for many years, was applied for simultaneous 225 nm DAD detection and 550 nm UV protein detection of gelatin contents in marsmallow sugars and capsules of pharmaceutical food supplements with combination of syringe pump system and reverse phase HPLC-DAD-UV unit and by using isocratic separation and constant flow from HPLC unit with 0.25 mL/min discrete syringe pump flow simultaneously during 15 minutes. Interestingly, if the gelatin content was from bovine or fish origin, a positive peak could be observed in HPLC-UV protein detection at 550 nm but if the gelatin content was from porcine origin, a negative peak could be observed in HPLC-UV protein detection at 550 nm. The detection limits of gelatin protein were quite high (LOD:0.4 ppm, LOQ: 1.3 ppm).

Keywords: *Halal Analysis; On-Line HPLC-DAD-UV; Gelatin Determination; Protein Assay with Henna*

Introduction

Gelatin, which has been used in many branches of the food and pharmaceutical industry, is a protein with animal or plant origin. Gelatin, an animal hydrolysis product and a commercially valuable natural colloid polypeptide, which is used for the applications of tissue engineering, cosmetics, food technology (confectionery, meat and dairy products), the pharmaceutical industry (tablet coatings, soft and hard capsules) and drug delivery systems, has a molecular weight of 100 - 200 kDa from denatured collagen [1]. Kinetic strength of gelatin extracted from avian sources, such as duck feet decreased with increasing amounts of sugars (sucrose and lactose) was detected using Fourier transform infrared spectroscopy and mechanical rheometer [2].

In high performance liquid chromatography analysis, the imino acid content for duck feet gelatin was slightly lower than bovine gelatin. Gels prepared from bovine gelatin had higher viscosity and darker color according to duck feet gelatin [3]. Fish gelatin, a class of biopolymer by hydrolysis of fish collagen, is used as a nutritional diet product due to its high amino acid content. Fish gelatin is an excellent diet product that is harmless to the body compared to the clinical treatment and medication of people with chronic diseases such as hypertension, osteoporosis and diabetes. Fish gelatin can be functionable as a biodegradable material that provides cells, genes and proteins to tissues [4]. The effect of gelatin blocks protected by a composite plate in minimizing the impact in the event of impact against human tissues was analyzed experimentally and numerically [5]. Soft sugars based gelatin and different sweeteners (maltitol, isomalt and stevia) were formulated by moisture content, water activity, tissue analysis experiments and characterized by high and low field nuclear magnetic resonance [6].

A pair of porcine specific primers that identify the mitochondrial sequence region for conventional PCR testing have been developed for the detection of porcine gelatin in commercial confectionery products [7]. In a study, pig and bovine gelatins were digested using trypsin enzyme and then subjected to LC-MS/MS analysis. Specific peptides were identified. Chemometric analysis was performed using classification, retention times and mass variables between pig and bovine gelatins [8]. In order to identify the gelatin origin commonly used in capsule form in nutritional supplements, ammonium sulfate precipitation was followed by separating pig and bovine gelatin with distinct protein bands in SDS-PAGE gel electrophoresis at 140 kDa and 110 kDa, respectively [9]. In other study, it has been shown that bovine bone gelatin and pig skin gelatin can be distinguished by their effects on calcium phosphate precipitation using a pH reduction method [10]. In halal-haram theory, which is a part of Islamic legal system, halal means permissible and legal, while haram means prohibited [11].

The term halalan-toyyiban means that a product is halal, contains healthy ingredients and does not pose any health risks when the product is used [12]. Halal law is particularly important for the identification of foods that are allowed to be consumed, as food intake directly affects human health and behavior [13]. The origin and the production process of the raw materials of cosmetics and health products are vital because of Islamic laws stating that every Muslim should only consume halal. Demand for halal pharmaceuticals and cosmetics is growing at an annual growth rate of 6.8% by 2024, with 2.4 billion Muslim consumers on the worldwide. Halal cosmetics should not contain ingredients derived from pigs, blood, human body parts, predators, reptiles and insects [14,15]. Standard and Metrology Institute for the Islamic Countries-Halal Cosmetics Requirements (OIC/SMIIC), engaged in the evaluation about the requirements for the certification of halal cosmetics is a guide organizations in Turkey [16]. Halal foods should be considered as foods that are allowed to be consumed by Muslims according to Syariah laws. The main sources of gelatin are pigs, cows and fish collagen, which are widely accepted in Halal, Kosher and Hindu foods. In a work, the halal trial methodology was optimized under pure and mixed gelatin matrices with a tetraplex PCR assay involving eukaryotic, fish, bovine and pig control for simultaneous detection of these species with a single, easy and reliable approach [17].

Henna and other Arab plant dyes have been used in the Middle East and North Africa for generations. There are comparative studies on the dyeing of protein fiber with henna and other Arab vegetable dyes to help develop natural vegetable dye applications in China [18]. Henna is very popular in the United Arab Emirates; it is a part of culture and traditions. Natural henna allergy is not normal; however, the addition of para-phenylenediamine, which is toxic to natural henna, increases the risk of allergic contact dermatitis [19].

This study is very valuable as it is important to determine the halal origin of gelatin in confectionery and pharmaceutical dietary capsules commonly used in the Muslim community. Gelatin content in confectionery or pharmaceutical food supplement capsules, and its halal or haram detection could be performed qualitatively and quantitatively with negative or positive peak forms simultaneously with developed on-line HPLC-DAD and combined post column UV henna protein staining methodology in the study.

Materials and Methods

Sample preparation

The jellied candies (Marsmellows) (80g) purchased from Migros stores in Turkey and empty capsules (Hardline ARG 1250, 120 capsules) (80g) of food supplements for athletes purchased from a private pharmacy in Turkey were separately heated in a glass flask containing 200 mL ultrapure water (obtained from Millipore water refinement system (Sartorius, arium pro-VF)) on a heating block (DynaBloc heating system, Thomas Scientific) until they were completely dissolved. Then four times of the cold acetone (-20°C) (Biomega, > 99 pure grade) volume was added to them. The mixture was vortexed (Velp Classic Analog) and kept at -20°C for 2 hours. The mixture was centrifuged for 30 minutes at 4000 rpm on the NUVE NF 400 Tabletop Centrifuge Instrument. The supernatant was discarded and the remaining of protein pellet was dried with air. Then, the pellet was dissolved in phosphate buffer solution (MERCK 107294, pH 6.88, di-sodium hydrogen phosphate/potassium dihydrogen phosphate) and protein extraction of all raw gelatins and products containing gelatin were

performed [20]. Protein concentrations of gelatins were measured as using BSA (Sigma-Aldrich, pH 5.2, ≥ 96%) standard in the ELISA instrument (novatek analytical systems) with the microplate reader by using the Bradford experiment [21].

Developed HPLC-DAD-UV system for halal protein detection with post-column henna staining

The Compact HPLC system was improved by unifying Shimadzu liquid chromatography (LC) Chrome LC-2010C-HT (Japan) including autosampler injection unit with a programmable single channel syringe pump (IPS 12-RS model, Inovenso laboratory devices, Turkey) that provides a wide flow rate range (0.732 µL/h - 4784.55 mL/h). The Compact HPLC system was contained of isocratic solvent (0.1M, pH 7 KH_2PO_4/K_2HPO_4 in aqua) manager pump, degasser, UV, deode array detector (DAD) and combined with second supported on-line reagent syringe pump (Figure 1). Data procurement (peak height, peak area, retention time) was executed by utilizing Shimadzu ChemStation Program. For protein fracturing, a purospher star reverse phase column with guard column (4.6 mm × 250 mm i.d., 5 µm particle size; Merck, Germany) was utilized and the injection volume of all samples was 50 µL. Analysis time was 15 minutes. DAD signal was set at 225 nm (maximum absorption for the gelatin protein) and UV on-line protein detection signal was set at 550 nm (maximum absorption after the post column reaction of henna and gelatin samples (halal and haram)). The flow rate of the syringe pump was set at 0.25 mL/min (optimized value) for loading henna staining reagent (prepared with isobutanolic infusion and filtration). Column temperature was set at room temperature, 25 - 38°C.

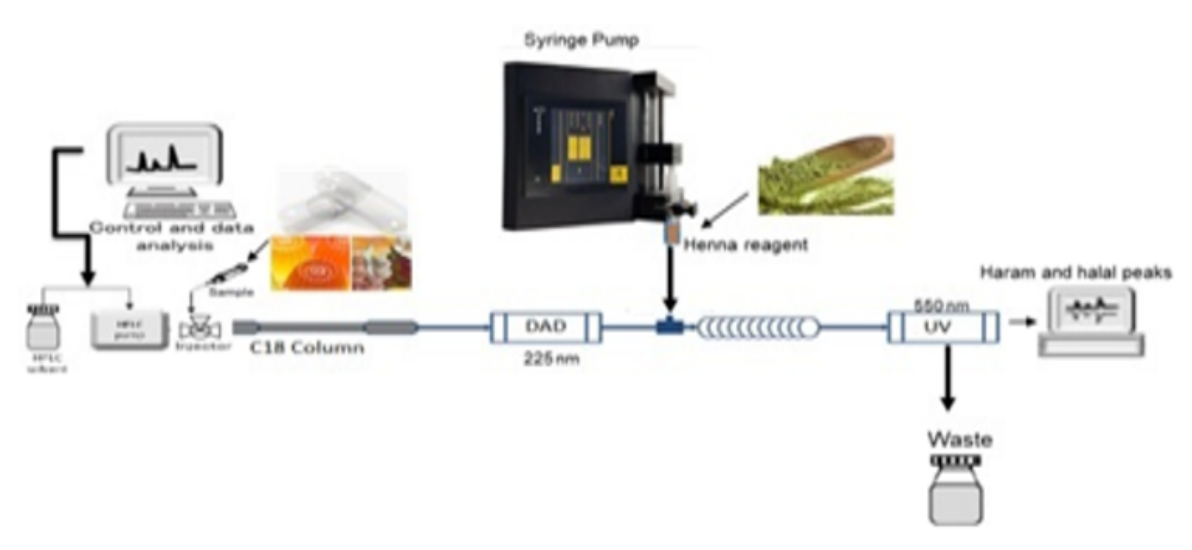

Figure 1: *Developed on-line HPLC-henna staining halal gelatin protein detection methodology.*

The reaction coil, which made of polytetrafluoroethylene (PTFE) tubing (0.25 mm i.d.), was adjusted as 1.5 m length (optimized value). Before HPLC analysis, all gelatin protein samples were filtered through 0.20 µm filter (agilent trademark) for preventing of bacterial contamination. Seven2Go Advanced Single Channel Portable pH Meter (Mettler Toledo) was used for the pH measurements of HPLC elution mixture. While at the end of the running of halal gelatin samples, chromatograms with positive peak was obtained at UV 550 nm simultaneous with DAD 225 nm, at the end of the running of haram gelatin samples, a chromatogram with negative peak in addition to positive peak at DAD 225 nm was obtained at UV 550 nm (Figure 2).

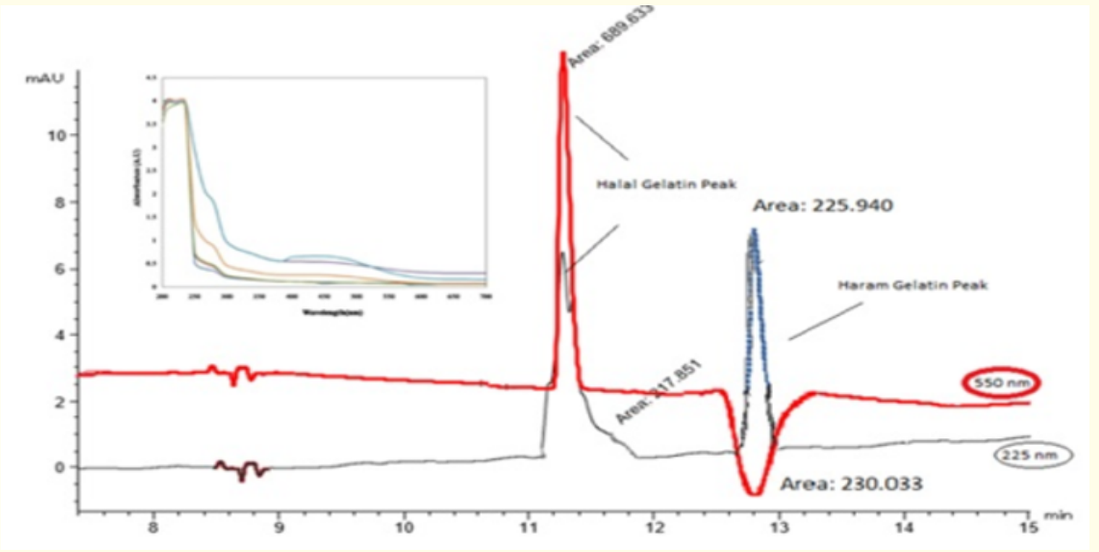

Figure 2: *Comparison of halal and haram gelatin peak choromatogram at DAD 225 nm and UV 550 nm.*

Determination of LOD and LOQ values for developed innovative optimized HPLC Halal protein methodology

Limit of Detection (LOD) and Limit of Quantitation (LOQ) are the analytical processing arguments of an analyte that can be accurately measured by the analytical procedure [22]. LOD and LOQ parameters were studied with the positive and negative peak profile fields for gelatin protein at DAD 225 nm and UV 550 nm by the originally developed post column HPLC-Henna protein staining method. The LOD and LOQ data of the 225 nm and 550 nm chromatograms were approximately calculated for the triple halal or haram peak detections in gelatin protein samples from different sources (sports food supplements and jellybeans) (Table 1). Gelatin protein samples were run for ten times in the on-line HPLC protein system to minimize the noise effect and obtain accurate results prior to calculations. The detection limits of the developed method about the halal analysis of gelatin protein were determined as 3 times and 10 times of the average standard deviation of noise. The developed post column HPLC-DAD-UV Henna protein staining method (for halal or haram peak separation) was optimized in terms of reactive flow rate, henna reagent concentration, tubing length and reaction temperature parameters [23]. Optimization was determined based on the maximum peak area values obtained by changing variable parameters in sequence and keeping other constant factors.

Experimental parameters	Optimized values	
λmax	DAD 225 nm	UV 550 nm
Concentration range	3.5 - 350 ppm	2.0 - 240 ppm
Linearity indicated by coefficient of correlation	0.9997	0.9998
Precision indicated by % RSD	<1.5%	<1%
Limit of detection (LOD)	0.6 ± 0.015 ppm (*)	0.5 ± 0.02 ppm (*)
	0.7 ± 0.025 ppm (**)	0.4 ± 0.01 ppm (**)
Limit of quantification (LOQ)	2.1 ± 0.045 ppm (*)	1.6 ± 0.01 ppm (*)
	1.8 ± 0.035 ppm (**)	1.3 ± 0.03 ppm (**)
Accuracy indicated by % Recovery	96.750 - 98.850	97.945 - 99.765
Retention time	11.5 ± 0.04 min	12.8 ± 0.04 min
Linear regression equation	y = 2.695x+1.536 (*)	y = 3.495x+0.446 (*)
	y = 2.521x+1.245 (**)	y = 3.575x+1.112 (**)
Tubing Length	2.5m	1.5m
SD: Average Standart Deviation, 95% confidence interval, critical ratio: p < 0.05, ppm: Parts Per Million, (*): For halal gelatin peaks, (**): For haram gelatin peaks		

Table 1: *Validation criteria of the developed on-line HPLC-DAD-UV henna protein staining method.*

Statistical data analysis

Statistical detection limit analysis was carried out by using Microsoft Office Excel (Corporation, 2017, Redmond, Washington, USA) and SPSS Version 21.0 software program. All statistical ChemStation peak area analysis data were reported significantly ($p < 0.05$) with standard deviation. Halal and haram peak area data were comparatively processed by ANOVA t test.

Result and Discussion

Some analytical techniques such as infrared spectroscopy, nuclear magnetic resonance spectroscopy, chromatographic based methods, electronic nose, differential scanning calorimetry, real-time polymerase chain reaction, enzyme-linked immunosorbent assay (ELISA), factor analysis to analyze pig derivatives, multivariate resolution analysis have been developed for halal analysis by using chemometric techniques such as artificial intelligence [24]. In a study, by using orthoftaldialdehyde, 4-chloro-7-nitro benzofurazan, 6-aminokinolil-N-hydroxysuccinimidyl carbamate as derivatizing reagents, differentiation of bovine, porcine and fish gelatin samples was performed by reverse phase HPLC amino acid analysis [25]. In this study, there was no need for derivatization, and the natural henna dyeing process of the gelatin samples obtained from different animal sources was performed by using on-line HPLC flow cytometry after column separation and determined as halal or haram for the Islamic society with positive and negative peak detection.

HPLC analysis shows that Henna extract contains a high proportion (57%) of colorant Lawsone compound [26]. When Henna applied to wool and nylon 6.6, Lawsone compound served as acid leveling because dye uptake increased with reduced pH and Lawsone compound changed the depth and color of the paints [26]. The linen fabric was dyed with a combination of henna and copper sulfate, and the obtained colored product was characterized by FTIR, TGA and SEM techniques. Henna staining was evaluated with functional properties such as antioxidant activity, antibacterial effect and UV protection [27]. Dyeing kinetics on protein fiber fabric of natural dyes extracted from henna leaves were studied. A systematic and cleaner production strategy was characterized with spectroscopic technique by using a henna dye bath to develop functional and colored linen fabrics [28]. The effects of active gelatin coated with henna (*L. inermis*) extract on beef quality were investigated during chilled storage. The effect of the beef coating prepared by using cuttlefish skin gelatin and henna juicy extract as a natural preservative was investigated during cooling of the meat on 1st, 3rd, 6th and 8th days. The combined coating reduced the total and psychrophilic vial bacteria count at the end of storage and extended the shelf life of the meat. The free amino acid content that formed faster in uncoated samples slowed down as the coating process reduced the rate of proteolysis. Lipid oxidation of the meat decreased during the storage period in the samples coated with gelatin-henna extract mixture [29].

Analytical derivatization in pre- or post-column modes have been one of the most widely used sample pretreatment techniques adapted to liquid chromatography [30]. Any derivatization reaction can be carried out in off-line or more preferred on-line mode [31]. A combined method has been developed by using on-line-HPLC-DPPH, two-dimensional liquid chromatography to simultaneously identify and separate antioxidants from *Lancea tibetica* which is the traditional medicinal plant [32]. Likewise, the on-line HPLC-ABTS method has been a convenient, effective and comprehensive to evaluate the contribution of polyphenols to the antioxidant properties of Chinese propolis [33]. Comparison of the performances of the cupric reducing antioxidant capacity test and the ferric reducing antioxidant power test was done on coffee and tea samples for analysis of antioxidants by using reaction flow chromatography, and the on-line CUPRAC method detected higher activity [34]. In addition, on-line protein analysis is one of the rising trends that have been used very effectively in recent years. For example; On-line protein isolation of allergenic components was performed by using multi-port valve key from soy flour samples with two-dimensional liquid chromatography and optimization of standard mixtures of glucose oxidase, myoglobin, immunoglobulin A, thyroglobulin and bovine serum albümin [35]. In one study, determination of the origin (beef or pork) of gelatin was intended. The gelatin was separated into peptides by enzymatic digestion and by using the mass spectra, pork gelatin could be distinguished from beef gelatin. But this was an expensive method [36]. In this study, halal or haram peak detection of gelatin protein was firstly and successfully applied and optimized with HPLC-DAD-UV application combined with post column syringe pump henna reactive dye flow and the results were

statistically significant ($p < 0.05$). Halal peak detection was simultaneously observed with positive peak profile and haram peak detection was simultaneously observed with negative peak profile. Optimization results were obtained as 0.25 mL/min henna isobutanolic infusion reagent flow, 50 mg/L reagent concentration, 28°C reaction temperature and 1,5 m tubing length by using a 0.5 mL/min HPLC stationary pump flow and gelatin protein at a constant concentration of 0.45 mg/mL (Table 1). The detection limits of the amount of gelatin protein measured in HPLC-DAD-UV by the protein-dye interaction with lawsone compound derived henna plant were quite good qualitatively and quantitatively (LOD:0.4 ppm, LOQ: 1.3 ppm) (Table 1). The UV spectrum of lawsone compound changed from 285 nm to 550 nm with batochromic shift and combined gelatin-henna compound was measured at UV 550 nm with on-line HPLC. This method was pretty cheap.

Conclusion

The cautious approach of the Muslim community to the use of food and food supplements with unknown animal source origin creates an environment of commercial insecurity. For halal certification, halal analysis is important. In this study, a new halal analysis method has been developed. Because animal resource has been important for the Muslim world in terms of haram and halal, determination of the kind of animal origin of gelatin has been valuable. The determination of porcine gelatin source of confectionery products and capsule medications which are considered as haram for the Muslim world has been successfully realized in a short time and with high sensitivity via this developed halal methodologic study.

In development of the method, protein analysis was done with henna painting technique, which has widely been used by the Muslim community, and it has been a very fast, cheap and dynamic system. When the peak chromatograms of halal and haram gelatin were examined, it has been understood that haram gelatin was more apolar. Halal gelatin according to haram gelatin had high amino acid content of arginine so the binding of halal gelatin with henna dye was stronger.

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Conflicts of Interest

The author stated that there is no conflict of interest relevant to this study.

Ethical Approval

This article does not contain any studies with human participants or animals performed by the author.

Informed Consent

Not applicable.

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