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APPLICATION OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) FOR DETERMINATION OF 1,3-DICHLOROPROPANE-2-OL IN FOOD MATRICES

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Key words**:** 1,3-dichloropropane-2-ol, HPLC, UV spectrophotometry, fluorimetry

This paper reports on preliminary results of quantitative determination of 1,3-dichloropropane-2-ol, one of the toxicants from the group of chloropropanols present in some foodstuffs, especially in soy sauce, by means of the high performance liquid chromatography. The aim of our study was to investigate and introduce a relatively inexpensive and simple method for screening of the studied analyte in food and environmental samples with the aid of the UV absorbing and fluorescent derivatives. The UV spectrophotometric and fluorimetric detectors were applied. The presented procedure could be applied in a number of cases for screening of suspected alimentary products before the use of more laborious and expensive GC-MS methods.

INTRODUCTION

1,3-Dichloropropane-2-ol (1,3-DCP) belongs to a group of chemical contaminants known as chloropropanols. 1,3-DCP, together with 3-chloropropane-1,2-diol (3-MCPD), were originally identified as contaminants of the savoury ingredient acid-hydrolysed vegetable protein, which is produced by treating proteins from hydrolysed vegetables, such as soya, with hydrochloric acid. In acid-hydrolysed vegetable protein, the components of fats and oils in the starting materials are chlorinated at high temperature to form chloropropanols. The acid-hydrolysed vegetable protein is a widely used ingredient of savoury food such as soups, prepared meals, savoury snacks, gravy mixes and stock cubes [Velisek *et al*., 1978; Collier *et al*., 1991]. 1,3-DCP and 3-MCPD were found in the acid-hydrolysed vegetable proteins [Min. Agric., 1993; FSA, 1999], and a range of other ingredients, most notably in soy sauce [Min. Agric., 1991; Crews *et al*., 2000]. Chloropropanols could come into sight in alimentary products during the storage of fatty foods with high salt content; in contact with packing materials, adhesives, vinyl resins stabilisers, epoxy resins and epichlorohydrin products, as well as in water as contaminants in polyamine flocculants used in the treatment of drinking water with chlorine, ozone and chloramine. Recently, Crews *et al*. [2001] suggested a considerable uptake of the concentration of chloropropanols as the effects of domestic cooking for a range of alimentary products. In 1991, the Committee of Carcinogenity considered 1,3-DCP to be a genotoxic carcinogen. Hodgson [2001] reported the belief of the joint FAO/WHO Expert Committee on Food Additives that processing of defatted vegetable proteins produced 1,3 DCP, which was found to occur less frequently than 3-MCPD, and was supposed to cause cancers in animals, and could damage genetic material. The carcinogenicity study has been reported, with 1,3-DCP administered in drinking water to rats and positive results have been obtained [Toxicity profile, 1999; CMCF, 2003]. Although no statutory levels for 1,3-DCP in food were established, this contaminant should not be present in the alimentary products above the limit of quantification expressed as 0.005 mg/kg. 1,3-DCP has been found to be mutagenic in bacterial and mammalian cell systems *in vitro*. In 1997, the Food and Drug Administration (USA) established specifications in the Food Chemicals Codex of 1 mg/kg 3-MCPD and 0.05 mg/kg 1,3-DCP in acid-hydrolysed vegetable proteins. In Poland, a decree of the Council of Ministry (dated on 4th of March 2003) was issued, based on the regulations of European Union and adopting its regulations [Decree of Polish Ministry of Health, 2003]. Recently, L'Huillier *et al*. [2002] demonstrated that 1,3-DCP was associated with major necrosis of the liver in humans. In humans and laboratory animals, 1,3-DCP is metabolised by cytochromes to a hepatotoxin 1,3-dichloroacetone. The authors suggested that 1,3-dichloroacetone could be embryotoxic at doses that do not cause adverse maternal hepatic damage. Preliminary results using chick whole embryo cultures indicated that 1,3-dichloroacetone had an inhibitory effect on the whole chick embryo development, and that embryonic-derived cells were sensitive to 1,3-dichloroacetone but not to 1,3-DCP, suggesting a potential teratogenic effect of 1,3-dichloroacetone.

Determination of 1,3-DCP at 10–100 mg/kg level has been reported by Van Rillaer *et al.* [1989] and Van Bergen *et al.* [1992]. Food Standards Agency, London, UK, [FSA, 2001] in-

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formed that 1,3-DCP was measured by an automated headspace procedure with gas chromatographic separation and mass spectrometric detection. The method, which incorporated a deuterium labelled internal standard, had previously been validated and shown to have a limit of quantification of 0.005 mg/kg. Quantification was based on a calibration series of 1,3-DCP standard solutions, equivalent to a range of concentrations 0.003 to 1.3 mg/kg 1,3-DCP, measured with each batch. Chung *et al*. [2002] presented a GC-MS analytical method for the simultaneous separation and quantitative determination of 3-MCPD and 1,3-DCP, at concentrations about the current action limits adopted by the international communities.

An appropriate method for the trace amounts determination of 1,3-dicholoropropane-2-ol (1,3-DCP) in alimentary products by means of high performance liquid chromatography requires the following steps to be realized: (1) a convenient sample preparation procedure attempting to the isolation and pre-concentration of a fraction containing the studied analyte; (2) a derivatisation of the analyte to form the UV absorbing, or preferably, fluorescent derivatives, in order to make use of the high sensitive detectors; (3) a chromatographic separation of the derivatized analyte from interferences in the matrix.

The sample pre-treatment was studied and reported by a number of contributors, *e.g*. Van Bergen *et al*. [1992], Meierhans *et al*. [1998], and Brendan *et al*. [2000]. The basic procedures included the liquid-liquid extraction carried out on a column filled with the modified diatomaceous earth or silica gel.

The detection of trace amounts of hydroxy compounds by the high performance liquid chromatography (HPLC) is neither easy nor sensitive enough and the derivatization of the analyte molecules is required to achieve sufficient selectivity. Imai *et al*. [1988] selected a number of pre-column derivatisation procedures of the UV-labelling of hydroxy compounds for the determination of alcohols by means of the liquid chromatography. Valuable procedures involved derivatisations with various reagents such as *e.g*. phenylisocyanate [Biorqvist *et al*., 1978], 3,5-dinitrobenzoyl chloride [Pivnichny, 1984] and 4-naphthalene-1-azo-(4'-dimethylaminobenzene)sulfonate [Wolski *et al*., 1986] for the determination of aliphatic alcohols with the UV spectrophotometry. On the other hand, the fluorescence derivatization has been found to be one of the most sensitive methods for the determination of biologically active substances at low concentrations [Motte *et al*., 1996]. Sensitive determinations of hydroxy compounds with fluorimetric detection after the pre-column derivatisation have also been reported. The procedure including a reaction with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate was evaluated for hydrophylic and volatile alcohols. You *et al*. [2001] reported that carbazole-9,N-acetic acid reacted with aliphatic alcohols in the presence of N-(3-dimethylaminopropylo)-N-ethylocarbodiimide hydrochloride and 4-dimethylaminopyridine, to form stable esters which were suitable for the more sensitive fluorimetric detection and provided an interesting procedure for indirect determination of alcohols. Compared with other methods for the determination of alcohols, this method offered an advantage that anhydrous reaction conditions were not required for the derivatisation or storage of the reagents, and the derivatisation procedure of alcohols with 9,N-carbazole acetic acid was facile, inexpensive and reproducible.

The aim of our study was to investigate and introduce a relatively inexpensive and simple analytical method for quantitative determination of trace amounts of 1,3-DCP in alimentary products and environmental samples with the aid of the high performance liquid chromatography (HPLC) and the UV spectrophotometric and fluorimetric detectors.

MATERIALS AND METHODS

Materials and reagents. The materials and reagents were used as follows: carbazole (pure), aliphatic alcohols C_1-C_5 (pure), diethyl ether (pure), acetone (pure), n-hexane (HPLC grade), N-(3-dimethylaminopropyl)-N-ethylocarbodiimide hydrochloride (pure) and dimethylaminopyridine were obtained from Polskie Odczynniki Chemiczne (POCh), Gliwice, Poland, acetonitrile (HPLC grade), ethyl acetate (purity p.a.), methanol and deionised water, (HPLC grade), ethyl ester of bromoacetic acid (pure), dimethylformamide (pure), 1-propanol (HPLC grade) were purchased in Sigma-Aldrich, Germany. 1,3-Dichloropropane-2-ol, with the purity exceeding 99.0%, was obtained from the Institute of Industrial Organic Chemistry, Warsaw, Poland. As the investigated samples two soy sauces were taken, the first bought in Leader Price, Poland, and a soy sauce Trung Thank, Vietnam, bought in the market. Standard solutions (100 ng/mL) of 1,3-DCP and the aliphatic alcohols from C_1-C_5 were prepared by dilution with acetonitrile of stock solutions of each alcohol (1 mg/mL) in acetonitrile. The reagent solution 4.5×10^{-5} mol/L was prepared by dissolving 1 mg of 9,N-carbazole acetic acid in 2 mL of acetonitrile and diluting to 100 mL with acetonitrile. Solutions of 5.0×10^{-5} mol/L 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide and 0.1 mol/L of 4-dimethylaminopyridine were prepared in acetonitrile, respectively.

Chromatographic equipment. A HPLC set used was furnished by Gynkotec (Munich, Germany) and consisted of a pump, a gradient generator, an automatic sampler, an UV-visible diode array detector (DAD), type UVD 340 S and Chromeleon 4.32 software; a hexa-port valve injector from Rheodyne (Rohnert Park, CA, USA), and a fluorescence detector type RF – 535 produced by Shimadzu, (Tokyo, Japan), were also used.

Sample preparation

Liquid-liquid extraction. The procedure of the liquid-liquid extraction was carried out in order to isolate and preconcentrate the analyte. Extrelut NT 20 columns (E. Merck, Darmstadt, Germany) were used. The sample volume of 20 mL was introduced into a high 250 mL beaker and 20 mL of 5 mol/L NaCl aqueous solution was added. The content was sonicated on an ultrasonic bath and afterwards introduced on the Extrelut column and remained to stay for about 0.5 h. The following fractions were obtained: the first fraction containing non-polar components was eluted with 50 mL of hexane, the second fraction containing chloropropanols was eluted with a 50 mL mixture of hexane and diethyl ether (9:1 w/w) and afterwards the third fraction containing chloropropanediols was eluted with 100 mL of diethyl ether. In a modification of this procedure, only two fractions were

eluted: the first fraction - by 50 mL of hexane (elution of the non-polar constituents) and the second fraction - by 50 mL of ethyl acetate (the fraction contained the sum of chloromonoalcohols and chlorodiols). The eluates were evaporated to 1 mL volume in a vacuum rotary evaporator under a nitrogen stream, and were subsequently dried with anhydrous Na_2SO_4. The esterification with 9,N-carbazole acetic acid was carried out after the filtration.

Derivatisation. The derivatizing agent 9,N-carbazole acetic acid is not available commercially at present. The synthesis has been performed according to You *et al*. [2001]: 4.1 g of carbazole was dissolved in 10 mL of dimethylformamide; then 5 mL of ethyl ester of bromoacetic acid and 4 g of pulverised KOH were added. The reaction mixture was heated by means of microwave radiation in a microwave oven 9 times repeatedly for 10 sec each, with power of 800 W. After cooling, 20 mL of 4 mol/L KOH solution was added and the precipitated carbazole was filtered out. Two mol/L of HCl were added to supernatant to obtain pH 2, and the precipitated 9,N-carbazole acetic acid was filtered and rinsed with the water:etanol mixture (9:1 v/v).

The reaction of esterification of 9,N-carbazole acetic acid with aliphatic alcohols and 1,3-DCP is presented in Figure 1.

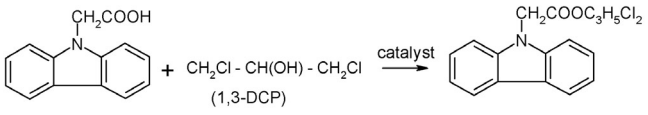

FIGURE 1. The esterification of 9,N-carbazole acetic acid with 1,3-DCP.

The esterification proceeded as follows: to 200 µL of a standard solution of the alcohol in a tube, 50 µL each of 1-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride, the coupling agent, 4-dimethylaminopyridine, the base catalyst, and 9,N-carbazole acetic acid, respectively were successively added. The tube was sealed and the mixture was heated at 60°C for 30 min in a thermostatic water-bath and then left to cool at ambient temperature. A 10-µL volume of the crude reaction mixture was injected into the chromatograph either directly or after the following purification step: to the crude reaction mixture, 5 mL of n-hexane was added. Then the mixture was washed successively with 5 mL each of 1mol/L hydrochloric acid, water, 1 mol/L sodium hydroxide and deionized water, respectively. The organic phase was separated and evaporated to dryness under a stream of nitrogen. Residue was redissolved in acetonitrile (100 µL), 10 µL of which was injected into the chromatograph.

HPLC analysis. The HPLC analyses were carried out on columns containing the C_{18}-chemically bonded stationary phases in the mobile phase gradient elution mode.

HPLC column: RP C-18, I.D. 4.6 mm; length 250 mm packed with wide-pore Octadecyl (C18), grain size 5 µm, was obtained from J.T. Baker, Chemical Company, Phillipsburg, New Jersey, USA.

Mobile phase composition: two initial solvent mixtures were used in the gradient elution mode: mixture A – composed of water-acetonitrile (65:35, v/v), with 0.1% acetic acid and 0.1% triethylamine at pH 6.5; and mixture B – composed of acetonitrile-water (95:5, v/v). An increase in mixture B content from 0 to 100% was performed during 20 min. The flow rate of 1.0 mL/min was maintained. Sample volume was 10 µL.

An UV diode array detector was used for detection at UV wavelengths of 254 and 330 nm. A fluorimetric detector was used at the following wavelengths: extinction at 330 nm, and emission at 360 nm.

RESULTS

The chromatographic separation of extracts containing 9,N-carbazole acetic acid esters of aliphatic alcohols and 1,3-DCP from the other components present in the reaction mixture of the studied samples were achieved by means of HPLC in the gradient elution mode. The chromatograms were recorded with the diode array detector at 330 nm and the fluorimetric detector. The retention factors k, calculated by the means of equation 1:

$$k = \frac{V_M - V_{solute}}{V_M} \quad (1)$$

where: k – retention factor of the solute; V_M – retention volume of a non-retained solute (the dead volume); v_{solute} – the retention volume of the solute substance.

The retention factors for esters of 9,N-carbazole acetic acid and 1,3-dichloropropane-2-ol, as well as C_1-C_5 aliphatic alcohols are presented in Table 1.

TABLE 1. The HPLC retention volumes V_r and calculated retention factors k, for 1,3-DCP and C_1-C_5 aliphatic alcohols obtained in the programmed solvent gradient elution mode.

Derivatized analyte (9,N-carbazole acetic acid ester of:)	Retention volume v_r (mL)	Retention factor $k_{isocratic}$
Methanol	3.01	1.57
Ethanol	5.57	3.76
n-Propanol	8.16	5.97
iso-Propanol	7.97	5.89
n-Butanol	11.18	8.56
iso-Butanol	11.03	8.50
tert-Butanol	10.98	8.48
n-Amyl alcohol	14.37	11.29
1,3-Dichloropropane-2-ol	16.07	12.2
Darbazole (non-derivatized)	7.83	5.83
9,N-Carbazole acetic acid (non-derivatized)	4.37	3.51

The quantitative detector responses, registered as the sum of impulses for the dilution series of 1,3-DCP in acetonitrile were determined by the two detectors used: the UV spectrophotometric (DAD), and the fluorimetric detector at the selected wavelengths for the peak height and peak area measurements, are presented in Table 2 and 3. respectively. The wavelengths of excitation and emission were determined experimentally by monitoring the detector response of the analyte solution *vs*. the

TABLE 2. Dependence of the peak height and peak area *vs*. the injected amounts of 1,3-DCP for the UV spectrophotometric detector.

Amount of 1,3-DCP injected (ng)	Peak area (Impulses)	Peak height (Impulses)
500.0	10 715	676
100.0	1 659	245
50.0	724	165
10.0	134	72
Soy sauce 'Leader-Price'	-	-
Soy sauce 'Trung Thank'	-	-
Estimated value of the maximum of detector response amplitude h_{max}	85	45

TABLE 3. Dependence of the peak height and peak area *vs*. the 1,3-DCP concentration for the fluorimetric detection.

Amount of 1,3-DCP injected (ng)	Peak area (Impulses)	Peak height (Impulses)
5000		
1000	446 683	1 330
500	588 843	1 555
100	891 250	1 702
50	901 370	1 629
10	645 654	1 273
5.0	354 813	1 064
1.0	51 286	606
0.5	17 378	388
0.1	2 238	105
0.05	891	56
0002	309	29
0.01	167	19
0.005		
Soy sauce 'Lieder-Price'	-	-
Soy sauce 'Trung Thank'	2 570	117
Estimated value of the maximum of detector response amplitude h_{max}	45	25

excitation light wavelength change, followed by corrections of the registered emission wavelength *vs*. the constant excitation wavelength. Relations between the detector response obtained with the UV-spectrophotometric detector (DAD) (the peak height and peak area respectively) *vs*. the amount of injected 1,3-DCP are plotted in Figure 2, and those corresponding to the fluorimetric detector are presented in Figure 3.

Figures 4 and 5 depict fragments of the above-mentioned plots measured in the range of low injected amounts of 1,3-DCP.

Estimations of the limit of detection (LD) and the limit of quantification (LQ) were carried out according to Caporal-Gautier *et al*. [1998]. The limit of detection (LD) could be defined as the lowest amount of the analysed compound in a sample that could be detected, but cannot be determined quantitatively as a

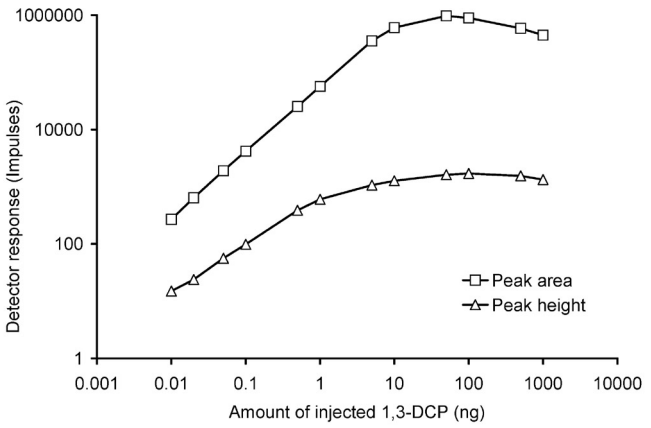

FIGURE 2. Plot of the UV-spectrophotometric detector (DAD) response *vs*. the amount of 1,3-DCP.

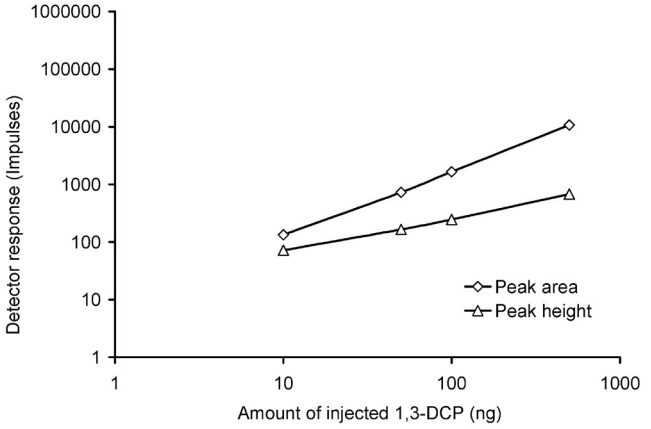

FIGURE 3. Plot of the fluorimetric detector response *vs*. the amount of injected 13-DCP.

precise value. The limit of detection could be expressed as a concentration, or as a mass amount and is derived from the lowest value X, which could be detected with a sufficient reliability for the given analytical method. If the analytical procedure gives a graphic report, *e.g.* a chromatogram, the limit of detection could be estimated from the registered noise level value. The maximum of the detector response amplitude h_{max} was calculated from the baseline of registered chromatograms in the area greater than half width of peak of the analysed compound peak. The detection limit could be assessed as follows:

$$LD = 3 \cdot h_{max} \text{ x } R \quad (2)$$

where R is the ratio: analysed compound mass *vs*. the detector response (given in the units of the chromatographic peak surface or the peak height); h_{max} - the maximum of the detector response amplitude obtained from the baseline of the chromatogram.

The value of LD, equal to 3 x noise level corresponds to a risk of 0.13% (the α error), if we accept the presence of the analyte in the studied sample. An opposite assumption, that the analyte is not in the sample, whereas in the reality it is present, is subjected to a high risk of 50% (the β error). In order to decrease the risk level, one can introduce higher value of R, *e.g.* if the indicator R equals 6, then the β = 0.13%.

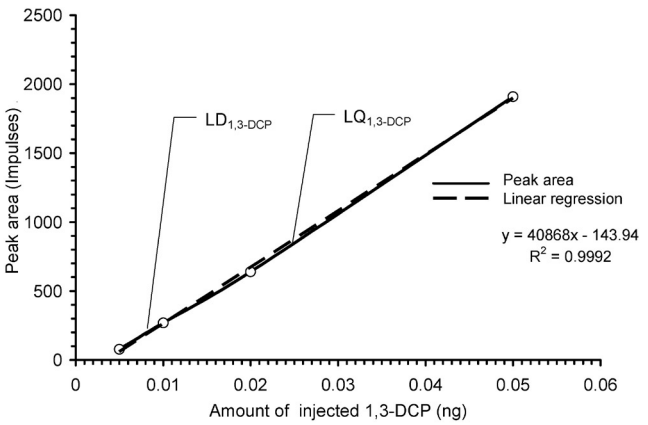

FIGURE 4. Assessment of the linear range of the UV spectrophotometric detector.

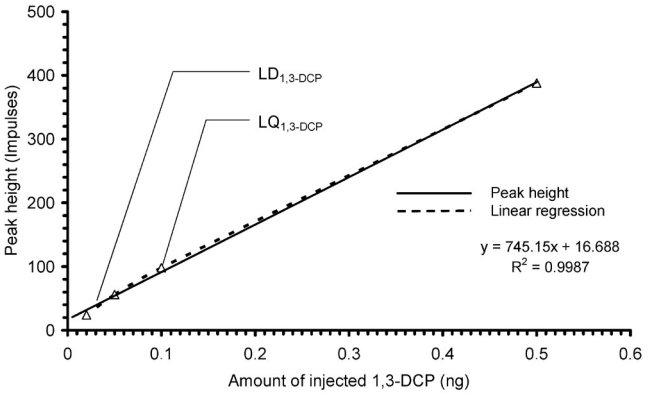

FIGURE 5. Assessment of the linear range of the fluorimetric detector.

The limit of quantification (LQ) is defined as the lowest mass or the concentration of an analysed compound that could be determined quantitatively with the declared reliability, precision and reproducibility, (equation 3):

$$LQ = 10 \cdot h_{max} \text{ x } R. \quad (3)$$

We used the graphic method of estimation of the LD and LQ. The obtained data are presented in Table 4.

Two chromatograms of the studied soy sauce samples with the fluorimetric detection are presented in Figure 6. The first sample did not contain the peak of 1,3-DCP, whereas this analyte was found in the second sample. The analysed soy sauce sample 'Thunk Thank' gave the peak area value of 2570 and the peak height value of 117 impulses corresponding to *ca*. 0.05 mg/kg sample. The studied soy sauce samples were analysed for the presence of 3-DCPD by means of a gas chromatographic method with the electron capture detector (ECD) described by Brendan *et al*. [2000] and modified and adapted by Kowalski *et al*. [2003] The first sample (soy sauce 'Leader-Price') did not contain chloropropanols, the second sample (soy sauce 'Thunk Thank' contained *ca*. 8.0 mg/kg of 3-MCPD.

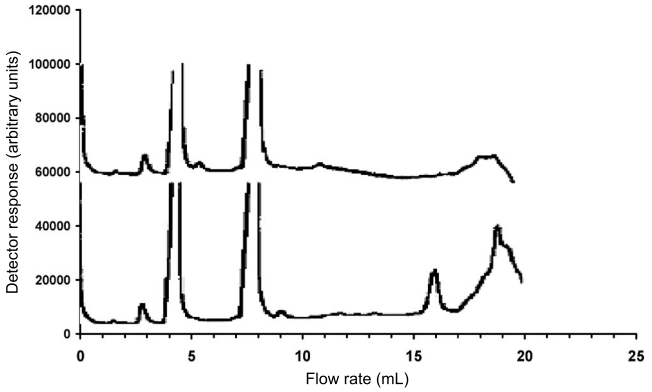

FIGURE 6. Chromatograms of the studied soy sauces: a) sample obtained at Leader Price, b) sample Trung thank, Vietnam.

DISCUSSION

No attention has been paid till now in the current literature to the application of liquid chromatography for the determination of chloropropanols. In this study, we proposed the application of the 9,N-carbazole acetic acid esters for determination of 1,3-DCP by means of HPLC with fluorimetric detection. The 9,N-carbazole acetic acid esters of 1,3-DCP could be prepared conveniently, *i.e*. in a relatively short time (0.5 h) and low temperature (60°C). The derivatisation reaction is only insignificantly influenced by the presence of humidity in the applied reagents, as well as in the sample. The 9,N-carbazole acetic acid esters of 1,3-DCP were obtained reproductively in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, the coupling agent, and 4-dimethyl-

TABLE 4. Estimation of limits of LD1,3-DCP detection, LQ1,3-DCP quantification and linear range of determination of 1,3-DCP with the UV-spectrophotometric and fluorimetric detectors.

Detector type	UV-spectrophotometric		Fluorimetric	
Procedure of determination	peak area	peak height	peak area	peak height
Value of the maximum of detector response amplitude h_{max} (impulses)	85	45	135	75
$LD_{1,3\text{-DCP}}$ (ng/10 mL injected sample)	0.45	0.75	0.008	0.030
$LQ_{1,3\text{-DCP}}$ (ng/10 mL injected sample)	1.5	1.8	0.025	0.10
Linear range (ng/10 mL injected sample)	1.5-200	2.0-200	0.025-5.0	0.10-1.0

aminopyridine, the base catalyst, in acetonitrile solution. The derivatives of 9,N-carbazole acetic acid are stable and show an important absorption in the whole UV region with a local maximum at 230 nm), as well as the fluorescence at 360 nm, after being excited by the UV light at 230 nm.

A comparison of plots of detector response *vs*. the amount of injected analyte 1,3-DCP indicated that the measurements of the peak area values gave steeper curves, *i.e.* the sensitivity of the 1,3-DCP determination was better. Nevertheless, the sensitivity of the fluorimetric determination was by *ca*. 10^3 better than in the case of the UV-spectrometric detector. The plot of fluorimetric detector response *vs*. the amount of 1,3-DCP is composed of two parts. The first part for low analyte concentration is linear (samples not exceeding 5 ng 1,3-DCP in 10 μL of the injected solution – in the case of the peak area measurements and those below 1 ng, in the case of the peak height determination).

In the second part of the plot, the fluorescence quenching processes increase with the increasing analyte concentration that the emission intensity decline rapidly. Under the presented conditions of the fluorescence measurements using the peak area procedure, the following limits were achieved: the limit of detection $LD_{1,3\text{-DCP}} \approx 0.008$ ng/10 mL injected sample and the limit of quantification $LQ_{1,3\text{-DCP}} \approx 0.025$ ng/10 mL injected sample. The range of linearity was assessed in the concentration region from 0.0025 to 5 ng/10 μL. The application of the procedure of peak height measurements gave slightly lower values, whereas the LD and LQ values obtained by means of the UV spectrophotometric detector were in a region of *ca*. 10^3 higher concentrations.

A possibility of improving these limits appears when considering the use of solvents specially purified for the fluorescence measurements.

CONCLUSIONS

The application of the HPLC in the reversed phase mode with the mobile phase gradient elution gave rise to the separation of the analyte (1,3-DCP) from the other substances presented in the studied samples.

The use of the UV spectrometric detector did not provide the satisfactory limits of detection and quantitation of trace amounts 1,3-DCP.

Taking into consideration a substantial pre-concentration of the studied samples during the liquid-liquid extraction procedure and low values of the limits of detection and quantitation, the application of fluorimetric detection for HPLC determination of 1,3-DCP appears to be promising.

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