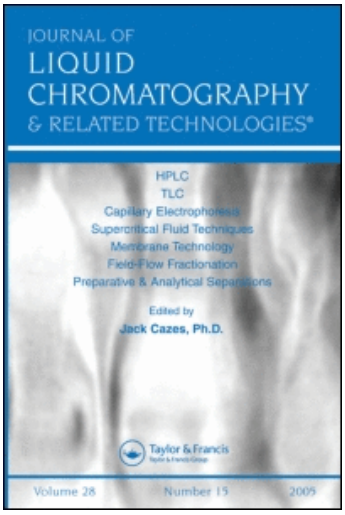

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Ramos, M. , Reuvers, Th. , Aranda, A. and Gómez, J.(1994) 'Determination of Chloramphenicol in Chicken Muscle by High Performance Liquid Chromatography and UV-Diode Array Detection', Journal of Liquid Chromatography & Related Technologies, 17: 2, 385 — 401
To link to this Article: DOI: 10.1080/10826079408013359
URL: http://dx.doi.org/10.1080/10826079408013359

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(2), 385-401 (1994)

DETERMINATION OF CHLORAMPHENICOL IN CHICKEN MUSCLE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND UV-DIODE ARRAY DETECTION

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ABSTRACT

A rapid and sensitive method for the determination of chloramphenicol in chicken muscle tissue is described. The method consists of mixing of the ground sample with anhydrous sodium sulphate and acetonitrile, followed by centrifugation and liquid-liquid partition of the supernatant with n-hexane. The acetonitrile extract is evaporated to dryness and the residue dissolved in dichloromethane. Purification was achieved by solid phase extraction silica cartridge and the chloramphenicol is eluated with a acetonitrile/water mixture (20%). Analysis is performed by high-performance liquid chromatography and Diode-Array detection where the peak's identity can be confirmed by comparing retention time and UV-spectra with external standard.

Linearity was studied up to 30 ng injected amount. Mean recoveries from spiked chicken muscle samples were 78.3% with a variation coefficient of 5.77%. The detection limit was 0.01 mg/kg. All the examined samples (50) proceeding from Spanish slaughterhouses turned out to be negative in chloramphenicol.

INTRODUCTION

Antibiotics have been widely used to treat human and animal diseases causing a lot of problems for the health authorities. These problems may have a toxic or allergic character caused by the accumulation of the residue in food for human consumption, but may also produce microbiological selection of resistent bacterial strains.

Chloramphenicol (CAP) has a broad spectrum activity and therefore has been used, not only in veterinary therapeutics, but also as feed additives. Since its introduction in 1949, adverse reactions and side effects of CAP have been reported over the last 30 years. The most known side effect is aplastic anemia, unrelated to dosage and irreversible. Others are blood dycrasias and bone marrow depression. To protect the consumer, zero tolerance levels have been proposed for CAP in edible tissues and milk (1) as the use of chloramphenicol in food producing animals is forbidden in various countries. In others, limits on CAP residues have been fixed. For example, the policy in several European Community countries is to reduce its use in food producing animals by setting limits on CAP residues in edible tissues. Countries, such as the Netherlands, have recently forbidden the suministration of CAP in leghen.

In order to monitor effectively the occurrence of residues, specific and sensitive analytical methods are

required. The microbiological methods have not the sensitivity necessary for the detection of these amounts. Radioimmnoassay has been used to identify and quantify chloramphenicol residues in food (2,3), which offers great sensitivity and may be used for screening purposes when a great number of samples has to be analyzed.

In the last years, various chemical detection methods for CAP in food have been published, generally based on chromatographic technics such as gas chromatography (4), liquid chromatography (5,6,7,8,9,10,11,12) and a colorimetric method coupled with thin layer chromatography (13). The most sensitive methods use gas chromatography with electron capture detection and tandem MS-MS (14) for the CAP residue determination in tissues, milk and eggs. However, gas chromatography involves derivatization of the sample extract, which may cause losses and lower recoveries.

Thin layer chromatography can be useful as a preliminary screening in the range between 10 and 1000 ppb (13). In the literature, several liquid chromatographic methods (5,12) are proposed based on reversed phase separation and UV and electrochemical detection. In previous work (15) we used the method proposed by Ellen and coworkers (16), but this method failed in the determination of CAP residues in fatty chicken tissues.

Recently new innovations in the purification step were introduced by Haagsma and coworkers (17, 18) using monoclonal antibodies in the clean up procedure.

In this work, we describe a HPLC method for the determination of CAP in fatty chicken tissue, based on the extraction of the tissue with acetonitrile, a medium polar organic solvent, followed by a solid purification step with a silica cartridge and finally separation and detection with reversed phase chromatography and Diode Array detection.

MATERIALS

Reagents

Water was purified by demineralization (Mili Q, Millipore). CAP was obtained from Fluka (Büchs, Switzerland).

Anhydrous sodium sulfate, sodium chloride and sodium acetate from Panreac (Montplet and Esteban, Barcelona, Spain). Ethylacetate, acetonitrile methanol, acetic acid (HPLC grade) and dichloromethane (for residue analysis) from Merck (Darmstadt, F.R.G.). Sep-Pak silica cartridges of Waters (Milford, USA) were used.

A CAP standard solution was prepared by dissolving 100 mg of CAP in 100 ml of methanol. Working standards for HPLC were prepared in the range of 100-600 ng/ml by diluting the standard solution with CH_3CN/H_2O (30/70).

The mobile phase solvent for HPLC was acetonitrile/diluted sodium acetate buffer (30:70 v.v). The diluted sodium acetate buffer was prepared by diluting sodium acetate buffer (1M pH 4.8) 1:100 with demineralized water. The mobile phase was filtered through 0.45 mμ Millipore filter membranes.

Apparatus

The instruments used were a Sorvall Omni-mixer (Duponts Instruments), an ultrasonic bath (Selecta, Madrid, Spain), a sample concentrator under a nitrogen stream (Techne Ltd, Oxford, Cambridge U.K.), a Macrotonic centrifuge (Selecta, Madrid, Spain), a rotatory evaporator (Büchi, Flawil, Switzerland) and a Moulinex homogenizer (Moulinex, Bilbao, Spain). The HPLC equipment consisted of a Waters pump (Mod. 6000 A), an injector (Mod. U6K), and UV absorption was monitoried at 278 nm with a Diode-Array detector (Waters 990). The HPLC column used throughout this work was a Nova-Pak 4 μ, C_{18} column in a Radial compression module of Waters (Milford, USA).

Flow rate: 1 ml/min., chart speed: 0.5 cm/min.

METHODS

Extraction and clean up of muscle tissue samples

Muscle tissue was prepared removing visible fat as far as possible and homogeneizing in a Sorvall Omni-mixer.

A 10 g sample of homogeneized muscle tissue was weighed into a centrifuge tube of 100 ml and thoroughly mixed with 30 g of anhydrous sodium sulfate and 30 ml of acetonitrile during 1 min in an Omni-mixer. The mixture was centrifuged during 10 min at 4000 r.p.m. and the upper organic layer transferred to a 250 ml round-bottom flask. The remaining aqueous phase was extracted two more times each with 30 ml acetonitrile. The combined acetonitrile phases were transferred to a separation funnel and extracted twice, shaking vigourously during 30 seconds, with two portions of 60 ml n-hexane. The hexane layers were discarded and the acetonitrile extract was transferred to a round-bottom flask and evaporated till dryness under a stream of nitrogen at 50°C.

Clean-up procedure

A silica Sep-Pak Cartridge (Waters) was washed respectively with, 5 ml of acetonitrile in water (20%), 5 ml of acetonitrile, 5 ml of dichloromethane and then dried by forcing during 30 minutes a gentle stream of nitrogen through the cartridge.

The sample extract was gently pressed through the cartridge with a disposable syringe, and the cartridge was washed with two 5 ml portions of dichloromethane.

After drying with a stream of nitrogen (about 30 min.), CAP was eluted from the cartridge with 5 ml

acetonitrile in water (20%). Then 1 ml of ethylacetate was added to the eluate and the mixture was shaken. The upper layer was transferred to a clean tube and the extraction with ethylacetate was repeated twice. The combined organic phases were evaporated till dryness in a sample concentrator at 50°C under a gentle stream of nitrogen. The dry residue was dissolved in 1 ml of the HPLC eluent.

Spiked samples

For the spiking studies, homogenized muscle tissue was used.

Ground tissue samples were spiked at levels of 10, 30 and 60 μg/kg at least 15 min before extraction according to the procedure described below.

Detection and Identification by HPLC

Different amounts of CAP were injected to check the linear response of the detector. Recovery experiments were carried out on spiked samples by injection of 50 μl of the sample extract, obtained under the conditions of extraction and purification as described above.

RESULTS AND DISCUSSION

Almost all the described methods for the HPLC determination of CAP residues use ethylacetate as the

extraction solvent. However, we observed an important co-extraction of fat using this solvent, causing a lot of troubles in the purification procedure. Therefore, we changed the extraction procedure of our previously described method (15) choosing a more polar solvent as acetonitrile, which has the same extraction efficiency for CAP, but is less active in the extraction of fat.

The fatty compounds were removed from the acetonitrile extract by liquid-liquid partition with n-hexane before the clean-up step with the silica cartridge.

Linearity of response, using peak height at 278 nm vs quantity injected, was studied by sucessive injections (n=2) of 50 µl alicuots of working solutions: 100, 300, 600 ng/ml corresponding to 5, 15 and 30 ng injected CAP respectively. The calibration graph was calculated using the method of least squares and can be expressed as:

$$y = 0.44\ x + 0.10$$

where y = CAP peak height in mm at 278 nm and x = the amount of injected CAP expressed in ng. The linearity was excellent with a correlation factor r = 1.000.

A typical chromatogram of 15 ng of CAP injected in the described conditions, is shown in Fig.1.

To check the validity of the proposed extraction and clean up procedure, various chicken tissue samples were homogenized and spiked with 100, 300 and 600 µg/kg of CAP respectively and the extracts analysed.

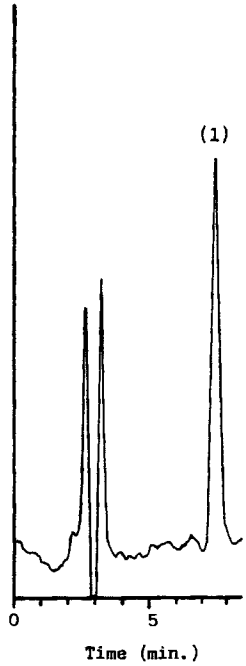

Figure 1 : Chromatogram of (1) 15 ng injected CAP in described conditions. (UV detection at 278 nm 0.005 a.u.f.s.).

Reagent blanks were extracted and analysed following the described method. Blanks are important as the influence of the surfaces of glassware may produce interfering peaks. Therefore, it is necessary to clean the used glassware by hand with a few drops of a cleansing fluid in water and rinse extensively. Alkaline soaps of the automatic dishwasher may cause undesired activation of the glass surface.

Fig. 2 shows a comparison of chromatograms of spiked (with 30 ppb CAP) and unspiked extracts of chicken muscle tissue.

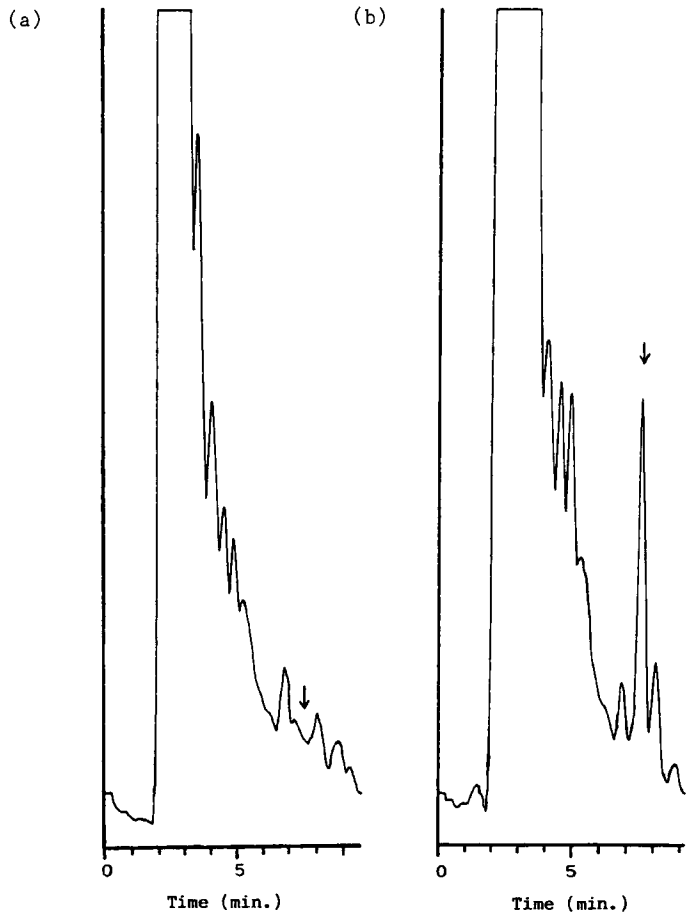

Figure 2 : Chromatograms of (a) unspiked and (b) spiked sample (30 ppb) of chicken muscle tissue (UV detection at 278 nm 0.005 a.u.f.s.).

TABLE I

Recovery of CAP in chicken muscle tissue

CAP added ($\mu g.kg^{-1}$)	% Recovery (n=4) mean	CV %
10	81.4	4.94
30	78.7	4.94
60	74.7	3.7

OVERALL = 78.3 %

Recovery results are shown in Table I. As can be seen, the recoveries are adequate in the range of the injected amounts with an overall recovery of 78.3% and CV of 5.77%.

A lot of 50 samples of chicken muscle tissue proceeding from different Spanish slaughterhouses was analysed with the proposed method.

Generally, peaks are identified by comparing the retention time with that of a standard. However, when working at trace levels, other compounds from the sample might have the same or similar retention times as the residues of interest. Therefore, an additional method for confirmation is required.

The Diode Array detector allows the possibility of getting this information about a compound as chromatographic and spectral data are both acquired and put into memory during the time of the run. These spectral data of an unknown peak can be used to confirm the identity of this peak.

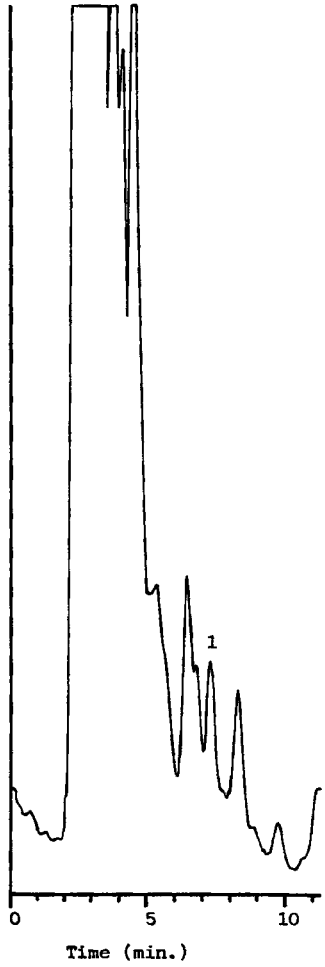

Figure 3 : Chromatogram of chicken muscle sample C 211 with a peak (1) at the retention of chloramphenicol.

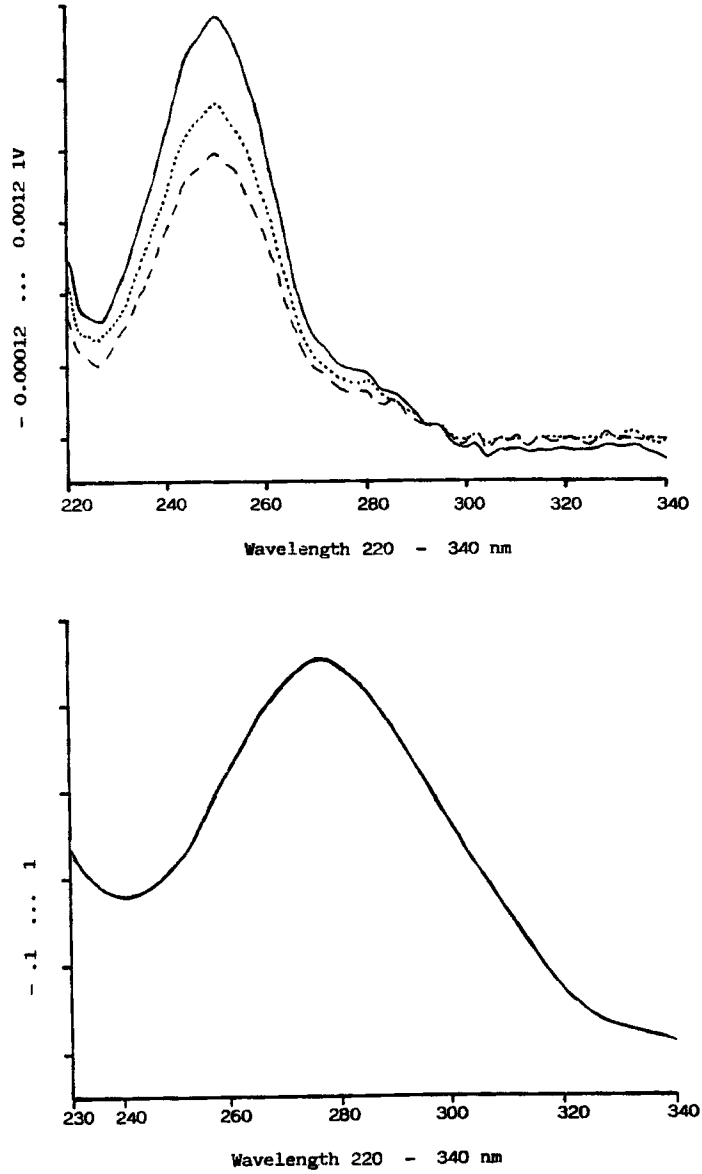

Figure 4 : Comparison of the spectra at different retention times of (a) peak (1) of chicken muscle sample C 211 and (b) external standard.

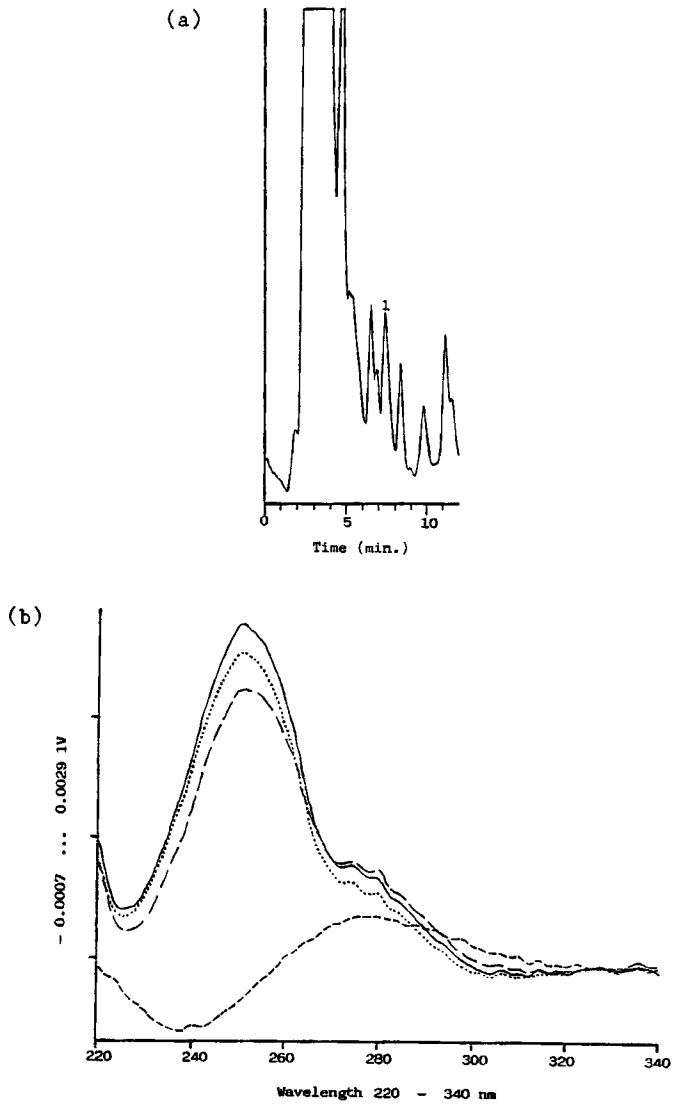

Figure 5 : a) Chromatogram of chicken muscle sample C 211 spiked with 5 ng chloramphenicol (UV detection at 278 nm 0.005 a.u.f.s.).

b) Spectra at different retention times of peak (1) of the spiked muscle sample C 211.

In neither of the analysed chicken samples could any detectable residues of CAP be detected. The detection limit was about 10 ppb; this concentration still gives well defined spectra. Moreover, the Diode-Array detector permits the "on-line" confirmation of suspicious peaks. For example, the chromatogram of sample C211 presents a peak at retention time 7.38 min, similar to that of CAP (Fig. 3). This unknown compound appeared to be a pure peak with a spectrum quite different to that of an external standard of CAP (Fig. 4a and 4b). Adding an amount of 5 ng of CAP to the sample, resulted in the appearance of the CAP spectrum in the downslope of the increased peak (1) as is presented in Fig. 5a and 5b.

Sometimes these interfering peaks were produced when samples underwent repeated thawing and freezing. So only once frozen samples must be used and thawed just before analysis.

In conclusion, the analysis of CAP by the reversed-phase clean up to separate the drug from the biological sample, is sensitive, specific and reproducible, as is demonstrated by the above mentioned results.

ACKNOWLEDGMENTS

The authors thank A. Gómez del Pino for technical assistance in the preparation of the manuscript.

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Received: February 8, 1993
Accepted: June 16, 1993