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Analysis of Chloramphenicol Residues in Indian Shrimps *Fenneropenaeus indicus*, *Penaeus monodon* and *Macrobrachium rosenbergii* by Liquid Chromatography–Tandem Mass Spectrometry

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ABSTRACT

Chloramphenicol (CAP) is a broad spectrum antibiotic which has been used in animal food production for a long time. The presence of CAP in commercial aquaculture shrimps in India was evaluated using liquid chromatography with tandem mass spectrometry. Method validation studies showed a 92 to 97% recovery. Over five levels of fortification in the marine shrimp matrix, the decision limit (CCα) and detection capability (CCβ) for CAP were found to be 0.198 and 0.295 μg kg^{-1}, respectively. Shrimp samples from major farms in three states (Andhra Pradesh, Tamil Nadu and Kerala) of India were screened for the presence of CAP and were found to be free of this antibiotic. The results indicated that all samples collected from the wild and aquaculture farms were free from CAP residues and thus safe for human consumption.

Keywords: antibiotic residues, LC-MS-MS, linearity, method validation, recovery

Abbreviations: CAP, chloramphenicol; **m-CAP**, meta chloramphenicol; **GESAMP**, Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection; **JECFA**, Joint Expert Committee on Food Additives; **MRL**, maximum residue level; **MRPL**, minimum required performance limit

INTRODUCTION

Animal husbandry for food production has always been dependent on various veterinary drugs, which provide numerous benefits related to animal health, welfare, and economic return for the industry. Since the benefits of sub therapeutic use of antibiotics in enhancing growth and feed efficiency in animals were first observed almost half a century ago, the number and use of these products has increased (Anon 1999). Chloramphenicol (CAP), a broad spectrum antibiotic, is widely used in aquaculture against various pathogens, especially to control those which are resistant to other antibiotics (Wang *et al*. 2004). A daily dose of CAP may lead to the development of non dose-related bone marrow depression (aplastic anaemia) in human beings (Rappeport and Bunn 1994; Roybal 1998; Greenwood 2000; Maluf 2002; Young 2002). CAP has been preferred by shrimp farmers since it has a long half life in solution and its optimal activity is between pH 7.4 to 8.0 (Ashwin *et al.* 2005). The elimination half-life ($t_{1/2}$ of CAP was estimated to be 10.04 h in *Penaeus chinensis* treated with medicated feed (Wang *et al*. 2004). The $t_{1/2}$ of CAP in soil at 25°C is 4.5 days; in pond water $t_{1/2}$ is 10.3 days at 25°C and pH 8, and 20.8 days at 37°C and pH 6 (JECFA 2004). It is reported that this antibiotic was in use among farming communities since 1950 (Alderman 1988). CAP is better than most antibiotics in terms of its ability to penetrate cells through cell membranes and can easily pass through infected materials entering organs. CAP interferes with protein synthesis in the invading microbes and kills them or prevents them from growing. As it is a very toxic substance, it is recommended that it should not be used in cases where the infections can be treated with other antibiotics (Okamoto and Mizuno 1962; Katzung 2006).

CAP has been banned in many countries including US, EU, India, Thailand and Singapore (Yogeswari *et al*. 2005) from use under any circumstance in food-producing animals because of possible residue carryover (Merck 1986). In view of the possibility of transmitting residues of CAP to human beings through aquaculture products, the use of CAP in aquaculture has been forbidden in the USA and in the EU. As there is no maximum residue level for CAP (EU 1994), the European Commission through EC directive 181/2003 established a minimum required performance limit (MRPL) for CAP detection in food products at 0.3 μg/Kg (EU 2003). In spite of this, it is reported that CAP is detected in several animal-derived foods as residues, especially in aquacultured products originating from Asiatic countries (Santos *et al.* 2005).

In India, presently over 185,000 ha is under shrimp farming. Small farmers, who have farms of less than 2 ha in size, form 90% of total aquaculture activities (Kumar 2007). The Aquaculture Authority, Government of India has banned the use of CAP along with other antibiotics and pharmacologically active substances for use in aquaculture (Pakshirajan 2002).

Various studies dealing with the estimation of CAP in different matrices using different methods were described previously (Gantverg *et al.* 2003; Impens *et al.* 2003; Mottier *et al.* 2003; Takino *et al.* 2003; Bogusz *et al.* 2004; Forti *et al.* 2005; Muñoz *et al.* 2005; Santosa *et al.* 2005; Ronning *et al.* 2006). However, a comprehensive documentation on the quality and quantity of various drugs applied in aquaculture in different countries is still lacking (Graslund and Bengtsson 2001; Johnston and Santillo 2002). Similarly, there are only few studies which address the issue of residues of chemicals present in the marketed products (Johnston and Santillo 2002).

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Original Research Paper

It is mandatory to check aquacultured shrimps, being a major item of export from India, for the presence of banned antibiotics. Therefore this study was undertaken to develop a new validated method to detect CAP and to assess the residual levels of CAP in aquacultured shrimps cultivated in the major aquaculture farms of India from January to June 2007 with the intention of evaluating their hazard level in relation to limits prescribed by importing countries.

MATERIALS AND METHODS

Chemicals and standards

CAP and m-chloramphenicol (MCAP) were procured from Sigma Chemical Co, St. Louis MO, USA. Water used for all analyses was from Milli Q water system (Millipore, USA). HPLC grade ethyl acetate, acetic acid, dichloromethane, acetonitrile and methanol were procured from Merck (Damstadt, Germany). All other chemicals were of analytical grade.

Preparation of standards

CAP standard stock solution of the strength 400 µg ml^{-1} was prepared by dissolving 20 mg of CAP in 50 ml methanol. The stock solution was stored at -20°C and was stable for 6 months. Different working standards were prepared fresh each time in the range of 0.1 to 1.0 ng ml^{-1} using 50% methanol in water and used for calibration. Fortification with CAP was done to marine shrimp samples by using a standard solution of 40 ng ml^{-1}. Marine shrimp samples were analysed for detection of CAP before fortification and were found to be free of CAP.

Preparation of mobile phase

Mobile phase A was 0.1% acetic acid and 10 mM ammonium acetate in water and mobile phase B was 0.1% acetic acid and 10 mM ammonium acetate in 95% methanol water. Mobile phases were filtered through a Whatman 0.45 µM membrane filter before use.

Collection of shrimp samples

Samples of cultured shrimp *Fenneropenaeus indicus*, *Penaeus monodon* and *Macrobrachium rosenbergii* were collected during harvest from ponds of Nellore and Bhimavaram, Andhra Pradesh; Chennai and Tuticorin, Tamil Nadu and farms at Kochi, Kannamali and Narakkal, Kerala. Wild samples of *F. indicus*, *P. monodon* and *M. rosenbergii*, caught by traditional fishermen from these three states of India were also included in the study. A total of 145 samples were analysed for the detection of CAP. For fortification studies, to 10 g of homogenized marine shrimp samples 25, 50, 75, 125 and 250 µl of 40 µg kg^{-1} standard solutions were added to obtain a final concentration of 0.1, 0.2, 0.3, 0.5 and 1.0 µg kg^{-1} of CAP. 150 µl of internal standard was added to all samples to get a concentration of 0.6 µg kg^{-1} of MCAP. The fortified samples were extracted and analyzed immediately after fortification.

Extraction of samples

Extraction was carried out as per Stuart *et al.* (2003) with slight modifications. Ten g of shrimps were homogenized in a corning centrifuge tube with an equal amount of anhydrous sodium sulphate using an ART-MiCCRa D8 homogeniser. 150 µl of internal standard and 20 ml of ethyl acetate were added. The tubes were agitated using a Vortex Genie mixer for 10 min, centifuged at 4065 × *g* (6000 rpm) for 10 min and the supernatant was collected. Extraction and centrifugation were repeated twice more with 10 ml of ethyl acetate. The supernatants were pooled and evaporated to dryness using a Heidolf rotary flash evaporator set at 45°C. The residue was reconstituted in 1 ml methanol and passed through an OASIS HLB 30 cc SPE conditioned with 1 ml methanol and then 1 ml water. The column was washed with 4 ml of 30% methanol and finally eluted with 4 ml methanol, evaporated to dryness and reconstituted with 1 ml of mobile phase B. Just prior to analysis, the sample was passed through a Whatman 0.2 µm syringe filter into an autosampler vial.

Sample analysis

Sample analysis was carried out using a Perkin Elmer HPLC with Series 200 autosampler, quaternary gradient pump (PE Series 200) and a PE 200 column oven coupled with an Applied Biosystems API 2000 tandem mass spectrometer with a turbo ion spray source. The separation was done using an Alltech Altima Rocket column (C18 RP 3 µm 7 × 53 mm). The oven temperature was kept at 40°C. The flow rate of the mobile phase was fixed at 0.3 ml/min. The injection volume was 30 µl and the run time was 15 min with a gradient of 10% B at 0 min and 90% B at the 15^{th} min. Each run was followed by a washing step with acetonitrile for 10 min. The turbo ion source was operated in negative ion mode at 400°C, curtain gas 30 psi, source gas 40 psi and collision gas 60 psi. As per EU decision 2002/657EC, four diagnostic ions are required for confirmation, the best results were obtained with 257, 194, 176 and 152, along with the parent (321), when the mass spectrometer was operated in multiple reaction monitoring (MRM) mode. For quantification purposes m/z 152 (CAP) and m/z 157 (m-CAP) were chosen.

Statistical analyses

In the present study, the method validation was conducted as per the EU decision 2003/181/EC (EU 2003). The decision limit (CCα) and detection capability (CCβ) were calculated by applying a calibration curve procedure as described in the 2002 EU directive. CCα is defined as the lowest concentration level from which it can be decided when the identified substance is present with a statistical certainty of 1-α. CCβ is the smallest concentration of the analyte that can be detected, identified and quantified with an error probability of $\beta \leq 5\%$. The external calibration curves obtained by CAP and MCAP in the 0.01 to 5 ng/g range were linear with a correlation coefficient of $R^2 > 0.998$ and $R > 0.999$. Data were expressed as mean ± standard error (SD). Student *t*-test was used for the statistical evaluation of the data. Significance was accepted at $p<0.05$.

RESULTS AND DISCUSSION

Chloramphenicol is an effective antibiotic that has been widely used since the 1950s to treat food-producing animals. As heavy side effects have been extensively demonstrated in humans (e.g. aplastic anaemia and hypersensitivity), the EC banned its use in food producing animals since 1994, in order to protect consumer's health (Forti *et al*. 2005). The use of liquid chromatography-negative ionization electrospray tandem mass spectrometry (LC-ESI-MS-MS) was proven as most selective and sensitive method for CAP determination in animal tissues. In the present investigation,

Table 1 Decision limit (CCα) and detection capability (CCβ) of CAP.

Criteria	Value (*n* = 25)
Decision limit (CCα)	0.198 µg/kg
Detection capability (CCβ)	0.295 µg/kg

Table 2 Measurement of repeatability of CAP in shrimp samples (*n* = 25).

Fortifiication levels of CAP (ppb)	Average value	SD	% CV
0.10	0.163	0.017	16.35
0.20	0.216	0.018	8.247
0.30	0.317	0.020	6.341
0.50	0.523	0.020	4.12
1.00	1.043	0.036	3.49

Table 3 Recovery of CAP from fortified samples (*n* = 25).

Matrix	Fortification levels (µg/ kg)	% ± SD
Shrimp	0.10	92.87 ± 4.95
	0.20	96.24 ± 3.28
	0.30	97.60 ± 2.05
	0.50	97.24 ± 3.94
	1.00	96.48 ± 2.56

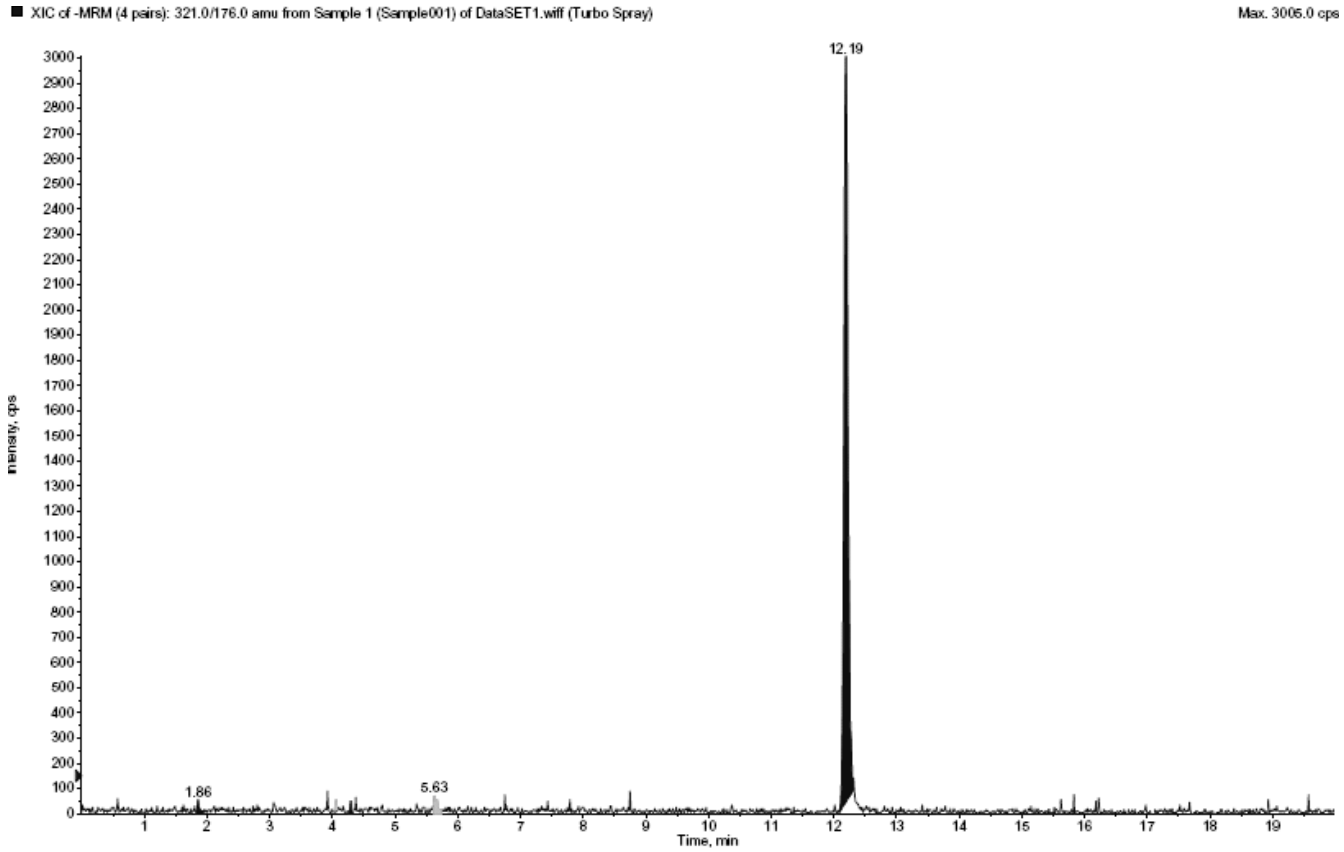

Fig. 1 Extracted ion chromatogram of CAP.

shrimp samples collected from aquaculture farms in three states (Andhra Pradesh, Tamil Nadu and Kerala) of India were screened for the presence of CAP residue using LC MS MS in ion spray negative mode after subjecting the samples through solid phase extraction.

Previously, Mottier (2003) developed a confirmatory method based on isotope dilution LC–ESI-MS–MS to determine CAP in meat samples. Santos *et al*. (2005) reported a validated methodology for the identification and quantification of CAP residues in rainbow trout (*Oncorhynchus mykiss*). Fortii *et al*. (2005) developed an effective liquid chromatographic method with tandem mass spectrometric (LC–MS/MS) detection for the determination of CAP in honey. Impens *et al*. (2002) evaluated the efficacy of different methods (ELISA, GC MS and LC-MS and LC-MS/MS) for the determination of CAP. In the present study, an attempt has been made to detect CAP at 0.295 µg/kg level using LC-MS/MS. The analyte was quantified by LC–ESI-MS–MS operating in negative ion multiple reaction monitoring mode. In this method, Oasis HLB SPE was used for sample purification, which made this protocol more rapid than other methods. Yet, there is no documentary evidence so far regarding screening of shrimp samples for CAP in India by adopting LC-MS/MS.

In the present study, the method validation was conducted as per the EU decision 2003/181/EC (EU 2003). Accuracy and precision were calculated from the analysis of blank shrimp samples fortified with CAP at 0.10, 0.20, 0.30, 0.50 and 1.00 µg kg^{-1}. Since no MRL exists for CAP the decision limit (CCα) and detection capability (CCβ) were calculated (**Table 1**) by applying a calibration curve procedure as described in the 2002 EU directive. The external calibration curves obtained by CAP and MCAP in the 0.01 to 5 ng/g range were linear with a correlation coefficient of $R^2 > 0.998$ and $R > 0.999$. The present findings concurs with an earlier reported study (Guy *et al*. 2004), which showed that CCα and CCβ of milk were 0.02 and 0.03 µg/kg, respectively. Reports by Forti *et al*. (2005) indicated that in honey sample analyzed the CCα and CCβ were 0.07 and 0.10 µg/kg, respectively.

The measurement of repeatability for shrimp samples fortified with CAP is given in **Table 2**. Four fortification levels between 0.10 and 1.0 were prepared. A total of 25 measurements were obtained by analyzing the samples on three different days. No significant difference was noted between measurements ($p<0.05$). Equivalent repeatability data obtained also are listed. The coefficient of variation over three days was 17% or less. The modified method used for the detection of CAP showed acceptable recoveries using various prawn matrices as given in the table. The recovery ranged from 92 to 97.0% over five levels of fortification (**Table 3**). The present observation is in accordance with a previous reported study (Bogusz *et al*. 2004).

The number of daughter ions and their corresponding ratios has to be measured for confirmation of a veterinary drug residue and define them as identification points as per EU regulation 2002/657/EC (EU 2002). Moreover, as per FDA (2003) the relative abundance ratio of these ions should match with comparison standard within ± 20% for analytes with three or more daughter ions. Further, since CAP is considered as a group A substance as per EU guidance, for which there is no MRL, a minimum of 4 identification points is required for confirmation. In this study four ions were monitored (**Fig. 3**) and hence conformed to EU guidelines (**Figs. 1, 2**). The present result is in line with earlier reported studies (Gantverg *et al*. 2003; Forti *et al*. 2005).

Previously, antibiotic residues have been detected in a small proportion (8-9%) of tiger prawns tested in the UK (Willis *et al.* 1999). Of 204 prawns tested in 1994, one contained detectable oxolinic acid, one contained sulphonamide and 16 showed the presence of oxytetracycline. In a study by Australian authorities, samples imported from eight countries were monitored for 56 antimicrobials (Anon 2005). However, the results of the present study indicate the absence of CAP residues in shrimp samples cultured in India.

In conclusion, the results of analysis showed good recovery when spiked with standards. A recovery of 92-97% was obtained for CAP in all matrices. The purpose of the study was to evaluate the presence of CAP in aquacultured species of shrimps in India, which were meant for export.

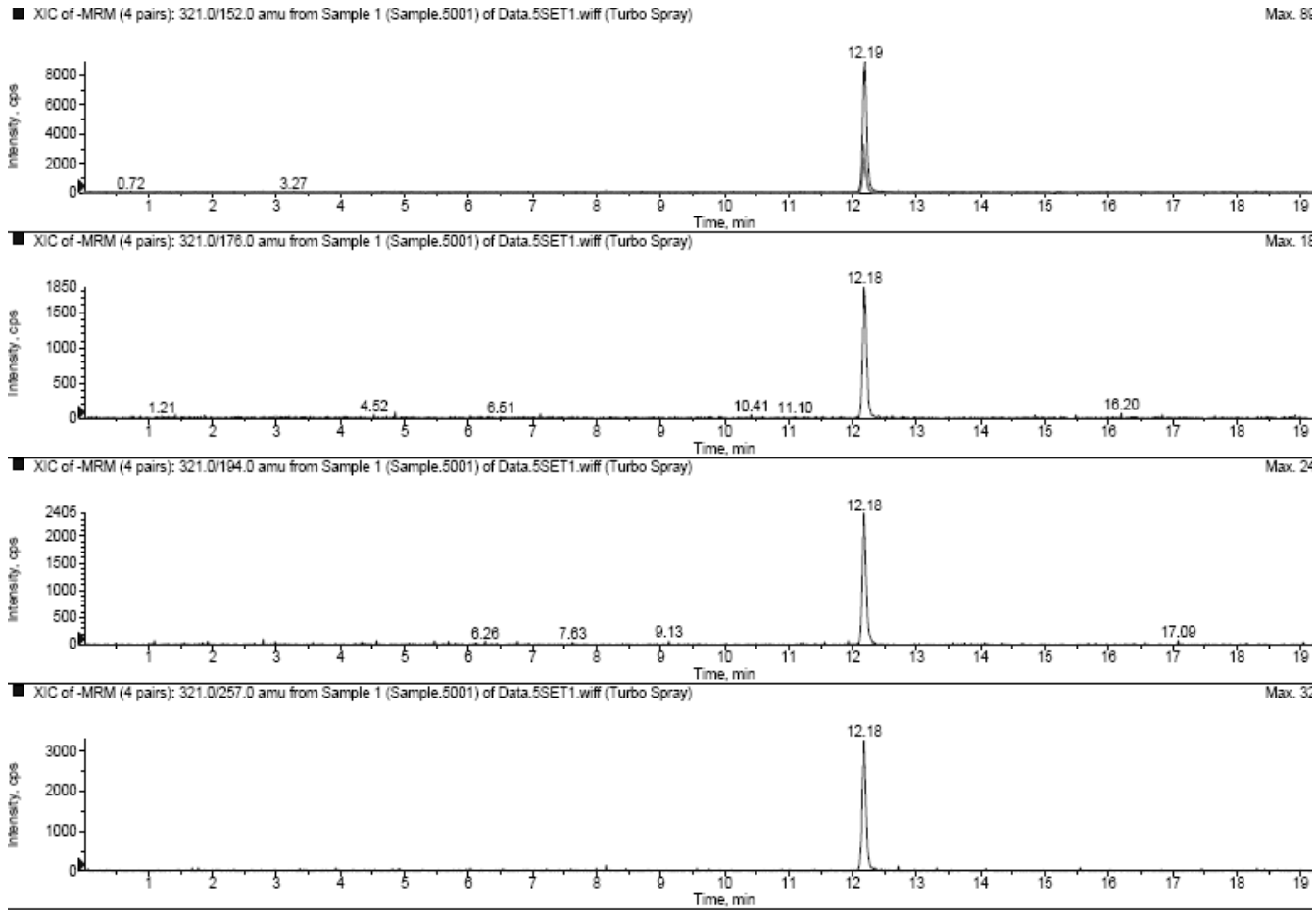

Fig. 2 Daughter ions 257, 194, 176 and 182 of CAP.

Table 4 Levels of CAP in shrimps collected from different farms in India.

Location of the farm	State	№ of samples	Result obtained
Nellore	Andhra Pradesh	24	Not detected
Bhimavaram	Andhra Pradesh	36	Not detected
Tuticorin	Tamil Nadu	12	Not detected
Chennai	Tamil Nadu	10	Not detected
Kochi	Kerala	28	Not detected
Narakkal	Kerala	15	Not detected
Kannamali	Kerala	20	Not detected

Control samples for the matrix did not show the presence of CAP. The results indicated that all samples collected from Nellore, Bhimavaram, Tuticorin, Chennai, Kochi, Kannamali and Narakkal were free from CAP (**Table 4**). The samples collected from the wild also were tested negative for CAP.

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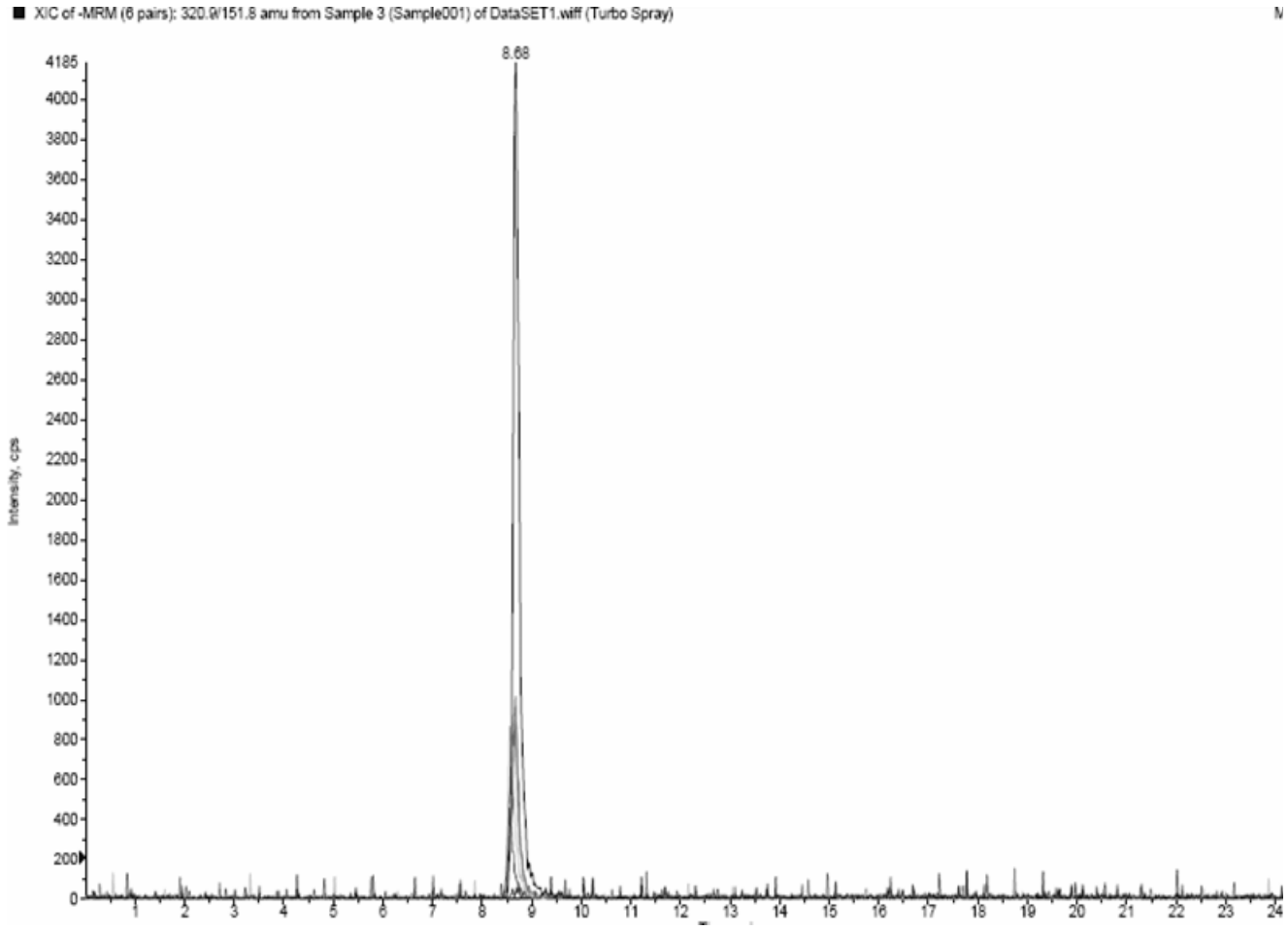

Fig. 3 Extracted ion chromatogram of m-CAP.

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