Chapter 7 Isolation of compounds

Because the optimal extract is intended for commercial application in the broiler industry, its activity should be ascribed to analytically determined active principles. The primary aim of isolation work was to isolate, determine structure and quantify activity of the major antioxidant compound that gave a deep red colour with vanillin-sulphuric acid reagent and had an R_f value of 0.84 in EMW solvent system.

Famakin (2002) isolated a stilbene, 2', 3', 4-trihydroxyl, 3, 5, 4'-trimethoxybibenzyl from the leaves of *C. woodii* that had high antibacterial activity. The R_f value of this compound on TLC developed in EMW system was 0.82 and very close to the R_f value of the target antioxidant compound (0.85 in EMW solvent system). In order to isolate the major antioxidant compound, serial exhaustive extraction was used as the first fractionation step, further fractionation and isolation was done using column chromatography.

7.1 Serial exhaustive extraction

Serial exhaustive extraction (SEE) is a mild technique that aims at fractionating a crude extract without introducing chemical changes. It was chosen as the first fractionation step in the isolation of the major antioxidant compound in the leaves of *C. woodii*. Because different solvents extract different phytochemical groups, serial extractions using solvents of varying polarities simplify fractions and enhanced the isolation of compounds from the complex crude extracts.

Three extraction series were adopted in preliminary serial exhaustive extraction; each series had solvents in a range of polarities varying from non-polar, intermediate polarity to polar solvents. In all cases, one plant sample was extracted successively with different extractants. The serial extractions were performed as follows:
Series 1: hexane, dichloromethane, acetone and methanol as extractants:
Series 2: hexane, ethylacetate, acetone and methanol.
Series 3: hexane, acetone and methanol

Ten g of leaf powder were extracted three times with each solvent by vigorous shaking in 100 ml of the various solvents for 30 minutes. The best serial extraction series was

determined based on the quantities extracted and the antioxidant activity present after each series had been completed.

Table 7:1: Amount in milligrams extracted from 10 g samples in the three serial exhaustive extraction series.

	Series 1	Series 2	Series 3
Hexane	258	285	293
Ethylacetate		628	
Dichloromethane	512		
Acetone	763	471	1243
Methanol	946	643	686
Total extracted	2479	2027	2222

Although the total quantity of material extracted in series 1 was higher than in the other two series, the acetone fraction in series 3 extracted the largest amount of the individual extractants in the various series.

7.2 TLC analysis

One hundred µg of each fraction was loaded on TLC (Merck, Kieselgel 60 F_{254}) plates and developed in the three solvent systems described in section 2.3. The plates were sprayed with vanillin-sulphuric acid reagent and 0.2% DPPH in methanol for visual detection of antioxidant compounds in the different fractions.

series of solvents described in 2.8.2 were used as eluents. This gave rise to 11 fractions from column elution when each of the solvent systems was used.

The different fractions resulting from VLC were not well resolved on TLC plates developed in EMW, CEF or BEA solvent systems and also to identify the solvent systems to be used in the next column separation on fraction TF2, it was necessary to develop a TLC system which would give good resolution of components in the fractions from VLC.

7.3.1 Development of a TLC separation system for column chromatography

Various TLC analyses were done on TF2 fraction in an attempt to determine an effective solvent system for column chromatography. One hundred µg each of the dried fractions was applied to the TLC plates for this purpose. Various ratios of different solvent mixtures were used. The following solvent systems were evaluated: chloroform/hexane; chloroform/ethylacetate; ethylacetate/hexane and chloroform/methanol combinations in different ratios, these solvents were chosen because of their varying polarities and selectivities (Snyder and Kirkland, 1979).

The ethylacetate and hexane combination in the ratio of 2:1 gave the best resolution and was chosen as the best solvent system for the next column separation.

test tubes. About 250 test tubes were collected and placed in a fume cupboard under a stream of air to concentrate the fractions. TLC analysis of column fractions was carried out with the intention to combine fractions with the similar compounds based on colour and R_f values.

7.3.3 TLC analysis of column fractions

Thin layer chromatography was carried out on the collected fractions to determine their complexities. After about 50% of the volume of the eluents evaporated, TLC analysis of every fourth test tube was carried out in EMW solvent system. Depending on the extent to which evaporation of the eluents in the different fractions had transpired 5–20 μl was applied on TLC.

Based on TLC analysis, the tubes were grouped into three as follows:

Group A: Test tubes 1–80
Group B: Test tubes 81–160
Group C: Test tubes 161–250.

Table 7:2 Amount in mg of grouped fractions obtained from column separations of TF2

	Test tube	Amount in mg
Group A	1-80	9
Group B	81-160	19
Group C	161-250	42

Mass spectroscopy confirmed the bibenzylic nature of the isolated compound. It gave a molecular ion at m/z 320 corresponding to $C_{17}H_{20}O_6$ and a major fragment at m/z 167 ($C_9H_{11}O_3$). The fragments are tropylium derivatives of the phenolic ring that is typical of bibenzyls (Letcher and Nhamo, 1972). This suggested that one ring contained two methoxyl groups and one hydroxyl group and the other one methoxyl group and two hydroxyl groups.

Table 7:3: ^{1}H-NMR (300MHz) and ^{13}C-NMR (75MHz) spectra data for isolated compound. Data obtained in $CDCl_3$

	Chemical shift (δ, ppm)		
Position	^{1}H	^{13}C	^{13}C (DEPT)
1	-	133.5	
2	6.39	56.2	CH
3	-	146.9	
4	-	132.3	
5	-	105.3	CH
6	6.39	56.3	CH
1'	-	121.6	
2'	-	142.1	
3'	-	132.8	
4'	6.55	145.3	
5'	-	102.4	CH
6'	6.35	120.2	CH
1a	3.8	36.5	CH3
1'a	3.8	32	CH3
3 OMe	2.82	76.6	
5 OMe	2.82	76.9	
4' OMe	2.82	77.4	

The exact positions of the hydroxyl and methoxyl groups around the two aromatic rings were ascertained from the chemical shift and the splitting patterns of signals of the aromatic protons. The isolated active compound was divided into aromatic rings arbitrarily labelled as 'A' and 'B' [Figure 8.11]. Mass spectroscopy of the isolated compound gave fragment of m/z 167 ($C_9H_{11}O_3$) representing aromatic ring A. It has two methoxyl and one-hydroxyl functions.

For ring A, ^{1}H-NMR showed a singlet at 6.394 ppm, which correspond to two protons. These are *meta*-coupled and magnetically equivalent and also imply that ring A is probably symmetrical. Therefore, the protons were placed at position 2 and 6. Positions 3, 5, 6 would have to be oxygenated as seen from the mass spectroscopic fragment m/z 167 ($C_9H_{11}O_3$).

The other fragment of m/z 153 ($C_8H_9O_3$) from mass spectroscopy represents the aromatic ring B. The proton at 6.55 ppm is *ortho*- coupled to that at 6.35 ppm. This is implied from the coupling constant J=8.4. The protons were placed at 5' and 6' leaving the other positions to be taken up by two hydroxyl and a methoxyl group.

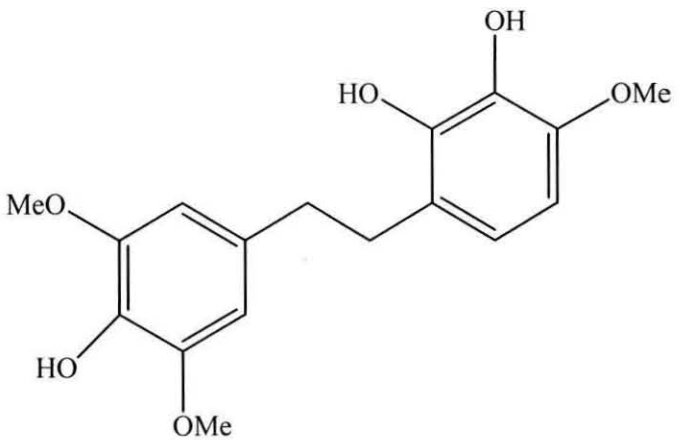

Figure 7:7 Structure of isolated active compound.

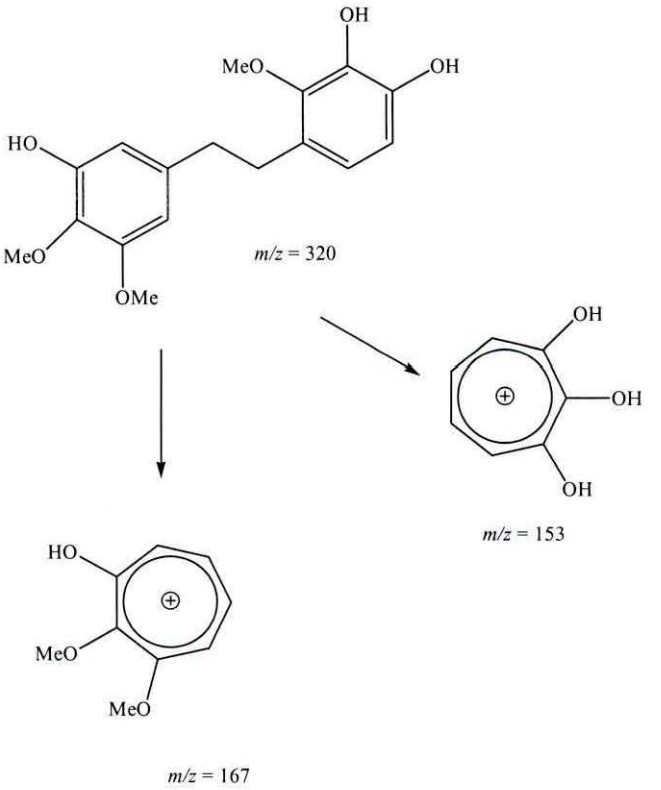

Figure 7:8 The isolated active compound and its fragmentation into two tropylium ions

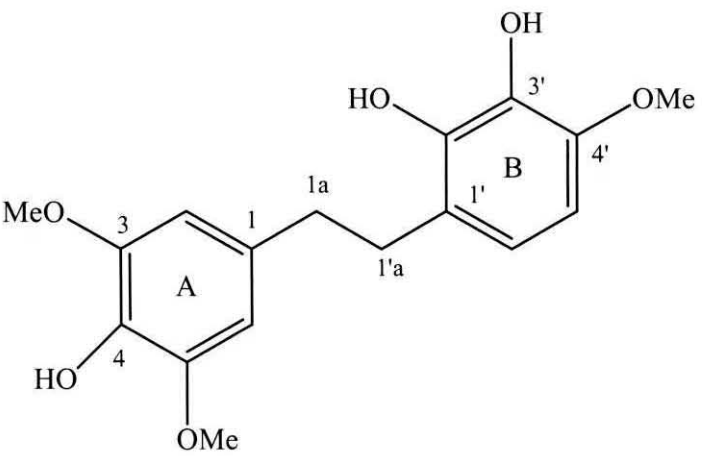

Figure 7:9 Isolated active compound with its two aromatic rings labelled as 'A' and 'B'

The isolated compound was proposed to be a stilbene, 2', 3', 4-trihydroxyl, 3, 5, 4'-trimethoxybibenzyl and identified as combretastatin B5 (CB5). This is the same as the major antibacterial compound isolated by Famakin (2002). This compound and its 2'-O-

glucoside have also been previously isolated from seeds of *Combretum kraussii*. Apart from C. *krausii*, this compound has not yet been found in any other plant. There are no previous reports on the antioxidant activity of this compound.

7.6 Antioxidant activity and cytotoxicity of combretastatin B5

The cytotoxicity and antioxidant activity of CB5 was quantified using the MTT assay on monkey kidney cell cultures and TEAC assay respectively. Stilbenes have been found in many families of higher plants such as Combretaceae, Liliaceae, Moraceae and Cyperaceae. They play important roles in plants especially in heartwood protection and in dormancy and growth inhibition. Certain stilbenoids, besides being toxic to insects and other organisms, have mammalian antifeedant and nematicidal properties (Croteau *et al.*, 2000, Gorham *et al.*, 1995, Schroder, 1999).

Stilbenes in general are known antioxidants whose occurrence in plants have been reported (Packer *et al.*, 1999 and Su *et al.*, 2002) mainly in grapes and wines (Burns *et al.*, 2002). The most documented antioxidant stilbene is resveratrol. In addition, stilbenes possess cyclooxygenase-I and-II (COX-1 and COX-2) inhibitory effects (Su *et al.*, 2002), as well as affecting lipid peroxidation (Stivala *et al.*, 2001), low density lipid (LDL) oxidation and vasodilation capacities (Burns *et al.*, 2002).

Combretastatins are stilbenes, dihydrostilbenes and phenanthrenes that have been isolated from the Combretaceae family (Petit *et al*, 1995). The most documented activity of combretastatins is their ability to cause mitotic arrest in cells in culture and to interact with tubulin, the major protein component of microtubules hence their wide use as anticancer drugs (Pettit *et al.*, 1987; Schwikkard *et al.*, 2000; Shnyder *et al.*, 2003), but there is no report in literature on the antioxidant activity of combretastatins.

7.6.1 TEAC assay of CB5

The TEAC assay was performed to quantify the antioxidant activity of combretastatin B5 as out lined in 2.6.2. A Trolox standard line was prepared by plotting percentage inhibition of the $ABTS^{+}$ radical against concentration of Trolox. The standard curve had a gradient of 155.89 and a percentage fit of 99.9%. A curve was also plotted for the isolated compound. The CB5 curve had a gradient of 1229.4 and percentage fit of 98.4%.

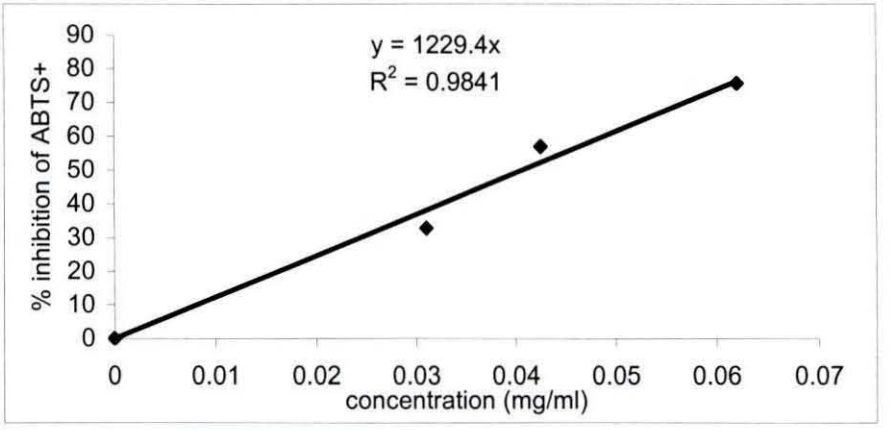

Figure 7:10: CB5 TEAC curve

The TEAC value of combretastatin B5 was calculated by dividing the gradient of its curve by the gradient of the Trolox curve to give a value of 7.886.

This result means that combretastatin B5 is 7.9 times a better antioxidant than the water-soluble vitamin E analogue (Trolox). This is the first report of the antioxidant activity of combretastatin B5.

7.6.2 MTT assay of CB5

In vitro cytotoxicity of CB5 was analysed using monkey kidney cells of the Vero type as outlined in 2.7.2 at 0.1, 0.01, 0.001 and 0.0001 mg/ml concentrations.
The Berberine chloride standard was also analysed at the same concentrations and gave an LC_{50} value of 3.002 µg/ml.

Table 7:4 Absorbance values at 540 nm for CB5 in the MTT assay

Log conc	Ave abs 540 nm	SD
-1	0.035	0.01
-2	0.537	0.08
-3	0.888	0.03
-4	0.906	0.05

In vitro cytotoxicity results of CB5 were analysed by plotting the logarithm of the concentrations against their absorbance values at 540 nm. The resultant curve had a percentage fit of 87.95% and the equation of the curve was, y = -0.2965(x) – 0.1497.

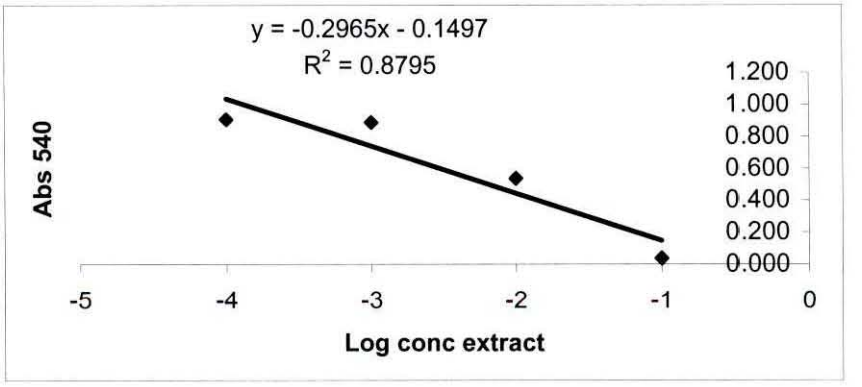

Figure 7:11 MTT assay curve for CB5

LC_{50} was calculated by substituting for y by half the value of absorbance at 540 nm for Berberine control (0.436). LC_{50} of CB5 was therefore 10.58 µg/ml.

7.7 Discussion

Bioassay-guided fractionation on silica gel 60 (63-200 um) in column chromatography resulted in the successful isolation of the major antioxidant compound present in the leaves of *C. woodii*. Combretastatin B5 had a TEAC value which was 7.89 times better than the water-soluble vitamin E analogue. The high TEAC value of CB5 is not surprising because most antioxidant compounds are polyphenolic compounds. The presence of many hydroxyl groups in the structure of CB5 accounts for its high antioxidant activity.

Combretastatins are aromatic compounds with antineoplastic and cell growth inhibitory properties. They cause mitotic arrest in cells in culture by interacting with tubulin, the major protein component of microtubules, at the colchicine binding-site (Pettit *et al.*, 1982). The MTT assay on monkey kidney cells gave an LC_{50} value of 10.58 µg/ml for CB5, a value that is similar to the reported LC_{50} value of the Berberine standard (10 µg/ml). The cytotoxic effect of Berberine is largely due to its antimitotic effect (Lin *et al.*, 1999, Fukuda *et al.*, 1999). It affects cell growth by interfering with spindle formation. The demonstrated cytotoxicity of CB5 could also be largely due to the same effect.

A major pitfall of the MTT assay seems to be restricted to polyphenols with antioxidant properties. At a lower, pre-apoptotic concentration range, these compounds, in spite of their growth inhibition properties, can induce in certain cell types an increase of the MTT-reducing activity that is not related to the number of living cells (Bernhard *et al.*, 2003). The result of this is the presence of an increased MTT-reducing activity in a slower growing cell fraction, compared to the faster growing untreated control cells.

Unfortunately insufficient material of CB5 was available to test its activity on bacteria isolated from poultry to determine to what degree the antibiotic activity of the optimal extract could be ascribed to CB5. Famakin (2002) found that the MIC value of CB5 against *S. aureus* was 16 μg/ml, *E. faecalis* was 125 μg/ml, *P. aeruginosa* was 125 μg/ml and *E. coli* had an MIC value of 250 μg/ml. CB5s antibacterial activities against *S. aureus*, *E. faecalis* and *P. aeruginosa* were higher than chloramphenicol and ampicillin antibiotics.

There was insufficient CB5 available to carry out *in vivo* experiments on poultry; however, it may be possible to synthesize CB5 for future *in vivo* experiments. Based on results from *in vitro* studies, the ability of the optimal extract to replace AFAs remains a possibility and this was examined in the next section.

Chapter 8 Tolerance and productivity studies in chickens

8.1 Introduction

Plants have been widely used in ethnomedicine around the world. A multitude of plant compounds is readily available and already being used by farmers for medication. Commercial application of these plants could be in the near future a common place. Some of the antimicrobial remedies currently used in livestock production have been reported and are listed in Table 8.1.

Table 8:1 Herbal remedies of potential use as antimicrobial agents in animal production (Cowan, 1999; Tedesco, 2001),

Common name	Scientific name	Compound	Class	Activity
Luceme	*Medicago sativa*			Gram + bacteria
Aloe	*Aloe vera*	Latex	Complex mixture	Corynebacterium, *Salmonella*, *Streptococcus*, *Staphylococcus*
Eucalyptus	*Eucaliptus globules*	Tannin	Polyphenol terpenoid	Bacteria
Fava bean	*Vicia faba*	Fabatin	Thionin	Bacteria
Garlic	*Allium sativum*	Allicin, ajoene	Sulfoxides, sulfate terpenoids	Bacteria
Green tea	*Camelia sinensis*	Chatechin	Flavonoid	Shigella, *Vibrio*, *S. mutans*, Viruses, Bacteria, Fungi
Oak	*Quercus rubra*	Tannins Quercitin	Polyphenols flavonoid	General
Onion	*Allium cepa*	Allicine	Sulfoxide	Bacteria
Rosemary	*Rosmarinus officinalis*	Essential oil	Terpenoid	General

The optimal extract demonstrated low *in vitro* cytotoxicity in the brine shrimp and MTT assays with LC_{50} values of 863 µg /ml and 226 µg /ml respectively. However, these findings alone are not enough for one to draw a conclusion on the potential toxicity of the extract hence the need to do *in vivo* tests that in effect would also assess the possible

application of the optimal extract to replace AFAs (growth promoting additives) without eliciting toxic effects.

Although plant extracts from the *Combretum* spp. have been effectively and safely used in human medicine both therapeutically and prophylatically (Hutchings *et al.*, 1996) and in ethnoveterinary medicine e.g. the use of galls from *Guiera senegalensis*, a member of the Combretaceae, to treat fowl pox virus (FPV) infections in chickens (Lamien *et al.*, 2004), there was a need to establish whether the target animals, the broiler chicken, could tolerate the optimal extract. Tolerance levels by broiler chickens to the extract were evaluated using the repeated dose toxicity procedure. The dosing regimens employed were inferred from *in vitro* studies since there are no recommended doses for *C. woodii* extracts in literature.

8.2 Method

In vivo toxicity studies were carried out as outlined in 2.9. All five treatment groups had 36 broilers except the 5 mg/kg treatment group that had 35 birds.

8.3 Results and discussion

8.3.1 Growth promoting effect

Due to the large variation in masses of the birds, there were no statistically significant differences in growth. This study detected no positive correlation in weight gain with amount of the optimal extract added in the finisher feed, thus the effect of the additives was not influenced by their concentrations in the diet. Compared to broilers diets without any feed additives (negative control) and diets incorporated with the antibiotic growth promoter bacitracin (positive control), the growth promoting effect of the optimal extract tended to be inferior throughout the dosing period [Figure 8.1].

However, the 2 mg/kg dose regimen of the optimal extract and the bacitracin positive control improved the Feed Conversion Ratio (FCR) of broiler chickens compared to the negative control. The improvement in FCR was larger with the former (6.2%) compared to the latter (1.7%). Five mg/kg and 10 mg/kg dose regimes of the optimal extract resulted in both, a reduced weight gain and FCR compared to the positive and negative control [Table 8.4]. It may be that the lowest dose of the optimal extract administered was already too

high. Throughout the 21 days dosing period until the last seven days, broilers tended to have greater improvements in weight when fed diets without any additives than diets with additives [Figure 8.1]. However, at the end of the 21 days dosing period, bacitracin tended to improve growth and the feed to weight gain ratio by 2.08% and 1.73%, respectively.

8.3.2 Health of the broilers

During the dosing period, none of the dose regimens of the optimal extract resulted in death or any visible toxic reactions in the broiler chickens. There was also no statistical significant decrease in the weight gain. Further analyses of the possible toxic reactions elicited by the optimal extract are investigated by post-mortem.

Table 8:2 Average weight of birds, standard deviation and variability over the 21 days dosing period

	Treatment group	Average mass of birds (g)	Standard deviation	Variability coefficient
From day 21	2 mg/kg	654	0.030	4.6
	5 mg/kg	624	0.032	5.1
	10 mg/kg	654	0.042	6.5
	Positive control	640	0.029	4.6
	Negative control	623	0.040	6.4
Day 28	2 mg/kg	1008	0.119	11.8
	5 mg/kg	964	0.080	8.3
	10 mg/kg	991	0.103	10.4
	Positive control	1027	0.082	8.0
	Negative control	973	0.076	7.8
Day 35	2 mg/kg	1390	0.069	5.0
	5 mg/kg	1358	0.057	4.2
	10 mg/kg	1387	0.080	5.7
	Positive control	1496	0.061	4.1
	Negative control	1420	0.077	5.4
Day 42	2 mg/kg	1822	0.184	10.1
	5 mg/kg	1726	0.149	8.7
	10 mg/kg	1783	0.111	6.2
	Positive control	1840	0.073	4.0
	Negative control	1848	0.109	5.9

Table 8:3 Average feed intake, weight gain and Feed Conversion Ratio (FCR) of birds in the different treatment groups over 21 days

	2 mg/kg	5 mg/kg	10 mg/kg	Neg control	Pos control
Feed intake over 21 days	66.258	64.919	71.028	72.681	72.798
Intake / bird over 21 days	1.8405	1.85483	1.973	2.01892	2.02217
Weight gain over 21 days	1.1682	1.10189	1.14013	1.20028	1.22486
FCR	1.5755	1.68332	1.73051	1.68204	1.65094

Table 8:4 Growth performance of broiler chickens in response to the optimal extract fed for 3 weeks, positive and negative controls. Values in brackets denote standard deviations.

Diet	Ave weight gain (g/bird)	Feed intake (g/bird)	% Improvement in weight gain	Food conversion (g feed/g gain)	% Improvement in FCR
Negative control	1200(0.184)	2019	-	1.68	-
2 mg/kg	1168 (0.184)	1840	-2.67	1.576	6.190476
5 mg/kg	1102 (0.149)	1855	-8.17	1.683	-0.17857
10 mg/kg	1140 (0.111)	1973	-5.00	1.731	-3.03571
Bacitracin (control)	1225(0.109	2022	2.08	1.651	1.72619

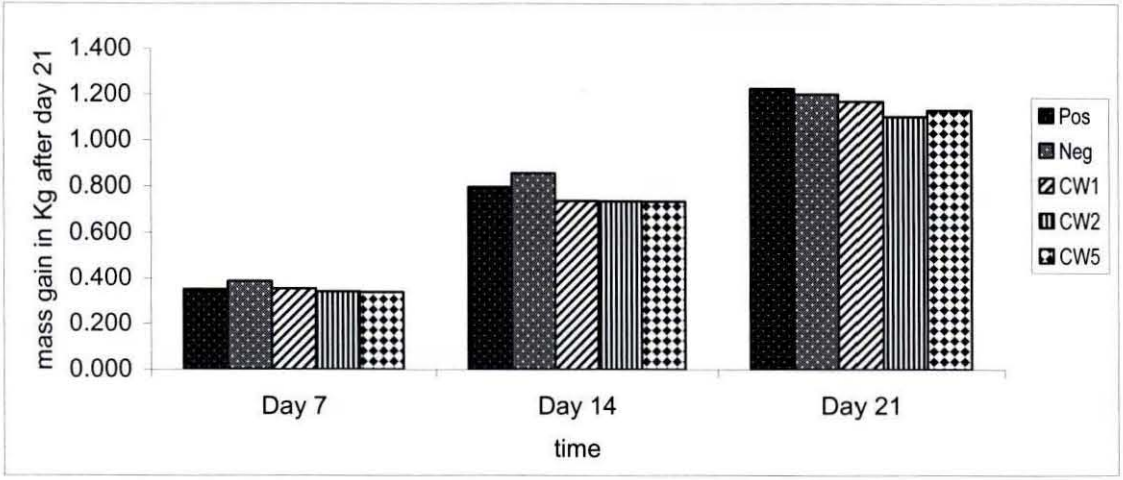

Figure 8:1 Weight gain after different periods of birds dosed with bacitracin (Pos), no feed additive (Neg), 2 mg/kg optimal extract (CW1), 5 mg/kg (CW2) and 10 mg/kg (CW5) optimal extract in feed.

8.3.3 Implications

The sustainability of broiler farming is dependent on both environmental implications and economic viability although these two factors are in direct conflict in many situations. Based on the assumption that the antimicrobial component of the optimal extract is novel and may readily control emergence of resistant strains of poultry pathogens, use of the optimal extract may be beneficial in the broiler industry if it becomes more effective as a growth promoter, no matter the benefits derived from its use in terms of controlling emergence of resistant pathogens. Based on the results presented in this work, there is room for eventual application of extracts from *C. woodii* in the broiler industry if methods that can improve extract potency are designed.

Because even the positive control had no statistically significant increase in productivity, the results probably mean that the hygienic situation under which the experiment was carried out did not lead to infection of the chickens. This experiment should be repeated on chickens challenged by prior infection in future experiments, possibly starting with a lower dose based on the feed conversion results [Table 8.4].

Chapter 9 General conclusion

Extracts of *C. woodii* leaves have *in vitro* antibacterial and antioxidant activity. Before investigating it in animals, attempts were made to increase the activities. After investigating a series of pretreatment and treatments, an extract with higher antibacterial and antioxidant activity (optimal extract) was developed by employing a single pretreatment extraction with hexane on *C. woodii* leaf material prior to extraction with acetone. This extract led to an improvement in antibacterial activity by 87.5% and antioxidant activity improved by 283.3% compared to the crude acetone extract. The optimal extract had a TEAC value of 2.3 in the TEAC assay; this result means it had 2.3 times the antioxidant capacity of vitamin E. Its average MIC against enteric poultry pathogens (*Campylobacter jejuni, Salmonella enteritidis, Clostridium perfringens* and *E.coli*) was in the order of about 0.1 mg/ml.

When the *in vitro* toxicity was determined, the extract was relatively nontoxic in both the brine shrimp assay (LC_{50} of 863 µg/ml) and MTT assay on monkey kidney cells (LC_{50} of 226 µg/ml). The improved extract therefore had a good activity and low levels of toxicity in *in vitro* studies.

Because the optimal extract was investigated for commercial application in the broiler industry, it was important to know the identity of the main antioxidant and antibacterial compound. Silica gel column chromatography was used to isolate the major antioxidant compound in the leaves of *C. woodii.* A stilbene, combretastatin B 5 was isolated as the major antioxidant compound. Its antioxidant activity was 7.9 times the activity of the water-soluble vitamin E analogue (Trolox). There are no previous reports of its antioxidant activity. This same compound has also been isolated from *C. woodii* by Famakin (2002) as the main antibacterial compound and from seeds of *C. kraussii* and has been found to have antimitotic properties (Pellizzoni *et. al*, 1992). The LC_{50} value of 10 µg/ml in the MTT assay could be ascribed to its antimitotic activity. Stilbenes are phytoalexins, these are antimicrobial compounds that accumulate in response to a pathogen (Kuc, 1990), and therefore the possible role of the bibenzyl in *C. woodii* is to protect the plant against any invading microorganism.

Preliminary studies and results from other researcher, (Eloff, 1999, Famakin, 2002, McGaw *et al.*, 2001) demonstrated *C. woodii* leaves to contain several antimicrobial and antioxidant compounds. Future work could be focused on identification and determination of the other active principles in the optimal extract for possible use as biomarkers in quality control. The study of the metabolism of these compounds and investigating the correlation between their activity and concentration may be useful. The successful isolation of one of the major biologically active compounds (combretastatin B5) from *C. woodii* leaves already provides a strong base for these inquiries.

In vivo toxicity studies of the optimal extract on broiler chickens demonstrated tolerance of the extract by the birds with no mortalities recorded during the dosing period and no bird showing behavioural signs of toxicity. There were no statistically significant differences between the growth promoting properties of the optimal extract and the positive and negative control. It will be worthwhile to determine the efficacy of the optimal extract as a growth promoter by repeating the experiment on chickens challenged with prior infection. This work should use lower doses of the optimal extract because 2 mg/kg dose regimen resulted in an increased Feed Conversion Ratio by 6.2 % compared to the negative control, a value that is about four percent superior to the value obtained in bacitracin-incorporated feed. If this observation can be confirmed under different conditions, this product may become a financially viable proposition.

The aims of the project have largely been attained and it appears that there may be scope for continuing work on plant extracts of *C. woodii*.

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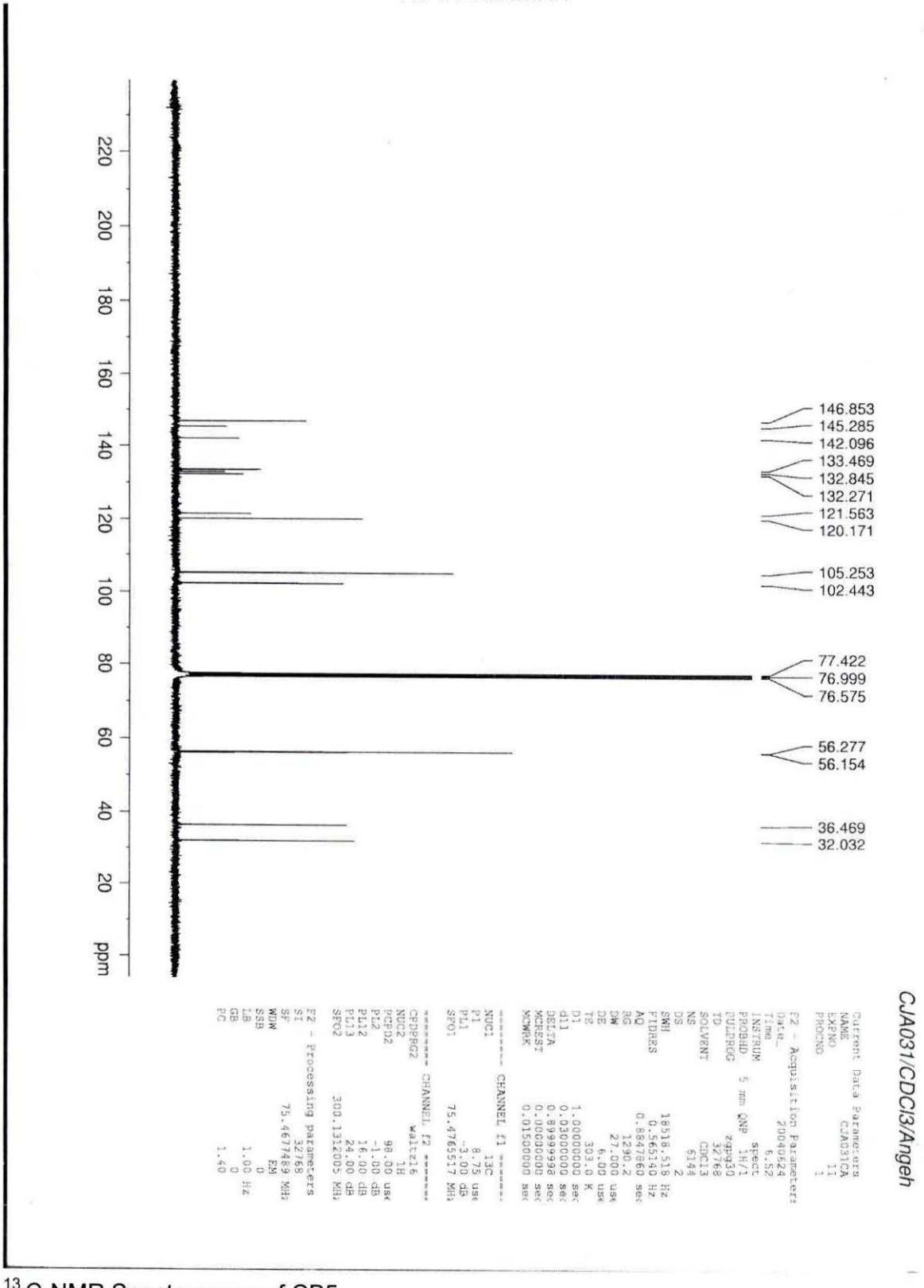
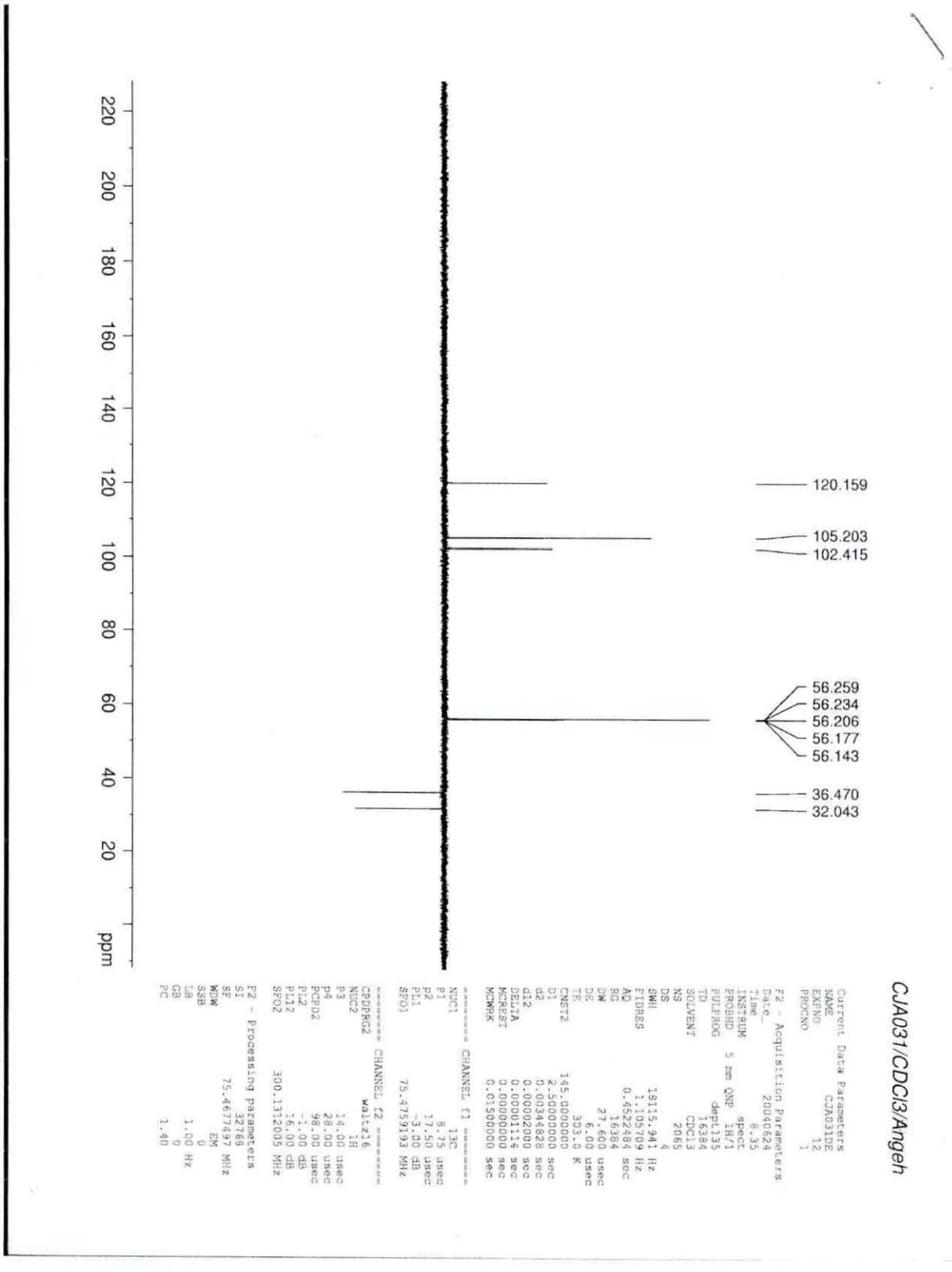
APPENDIX A



^{13}C-NMR Spectroscopy of CB5



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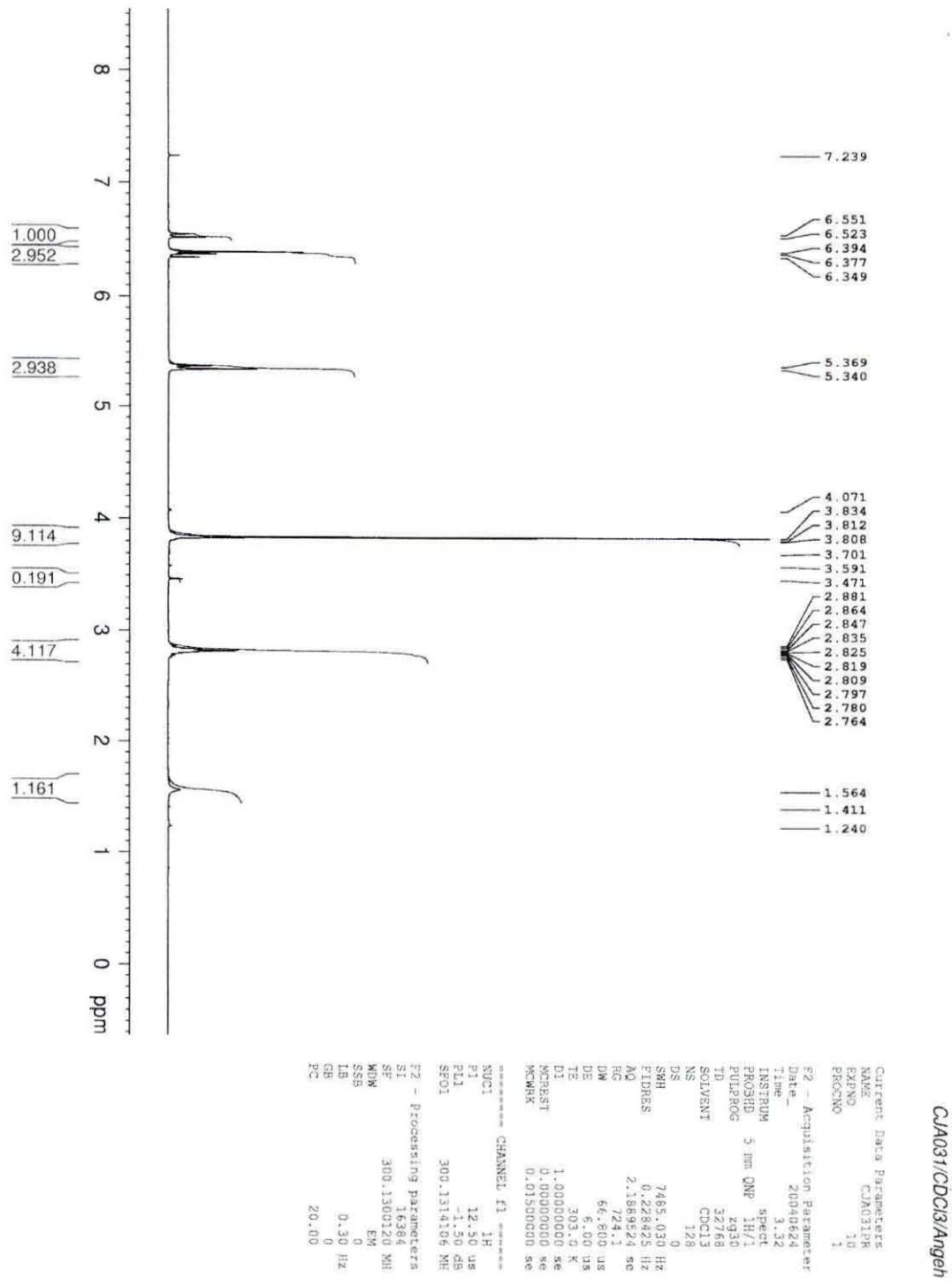

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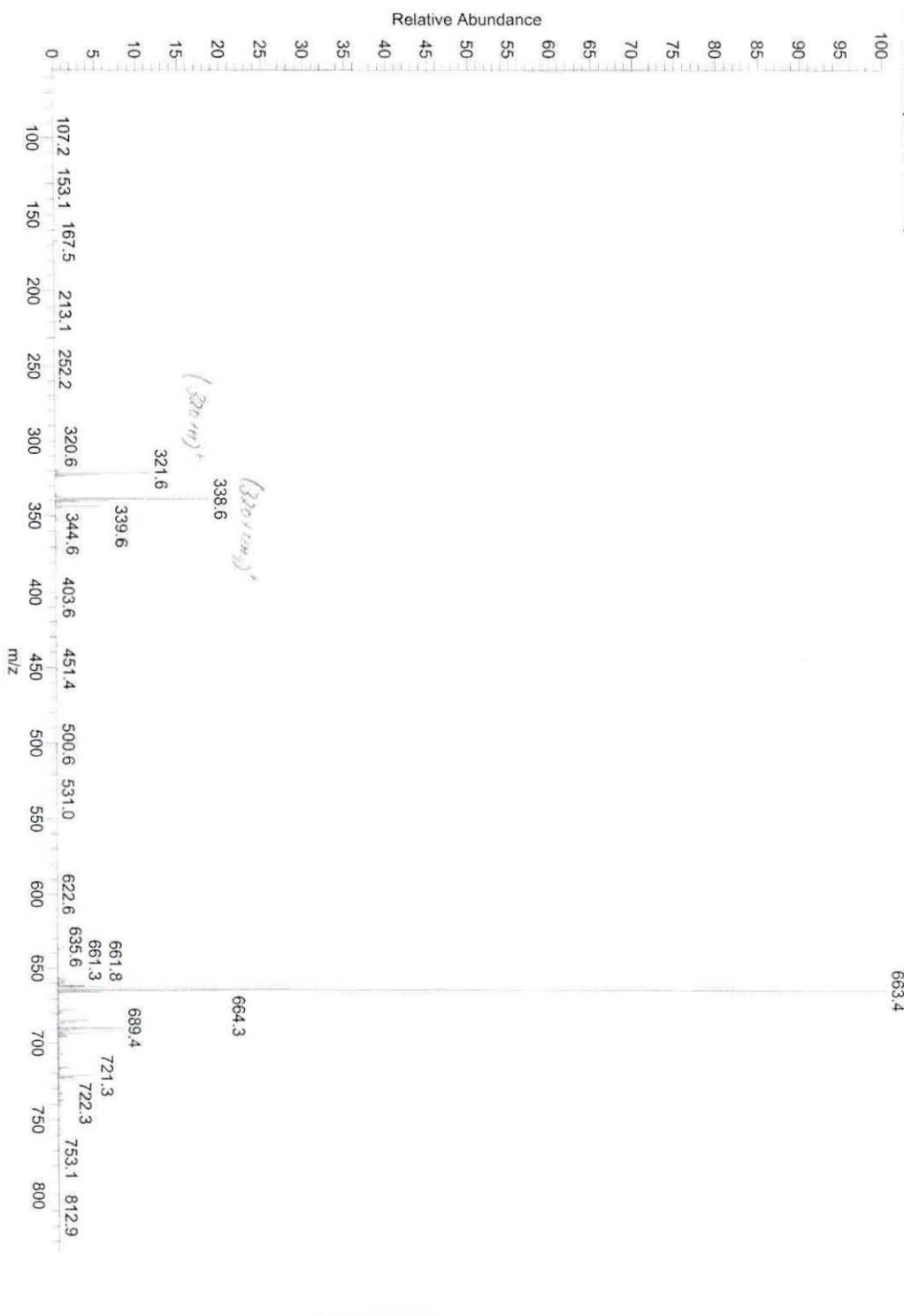

Mass spectrometry of CB5

APPENDIX B

Weight of birds in 2 mg/kg treatment group (A), 5 mg/kg treatment group (B), 10 mg/kg treatment group (C), negative control (D) and positive control (E) at day 21 (left) and day 28 (right).

	A	B	C	D	E	A	B	C	D	E
1	0.65	0.396	0.527	0.75	0.74	1.031	0.603	0.99	1.079	1.096
2	0.593	0.599	0.68	0.643	0.549	1.154	0.839	1.25	1.005	0.88
3	0.684	0.724	0.653	0.636	0.63	1.093	1.036	1.03	1.007	0.915
4	0.608	0.435	0.622	0.656	0.518	1.028	0.853	0.96	1.077	0.872
5	0.552	0.537	0.729	0.706	0.577	1.039	0.952	0.927	0.904	0.997
6	0.681	0.514	0.628	0.602	0.7	1.131	1.157	1.19	0.82	0.606
7	0.699	0.594	0.701	0.585	0.689	0.999	0.975	1.001	1.053	0.895
8	0.661	0.502	0.675	0.675	0.656	0.647	1.053	0.878	1.001	0.79
9	0.672	0.682	0.56	0.573	0.655	1.048	0.991	0.889	1.002	0.859
10	0.699	0.517	0.69	0.619	0.539	0.969	1.071	0.971	1.039	0.81
11	0.724	0.672	0.661	0.58	0.654	1.036	0.908	1.16	1.057	1.09
12	0.6	0.618	0.69	0.557	0.36	1.079	1.094	1.004	1.058	1.017
13	0.618	0.587	0.454	0.677	0.482	0.902	1.154	1.047	1.107	0.98
14	0.609	0.639	0.545	0.65	0.687	0.986	0.907	0.8	0.999	0.952
15	0.667	0.624	0.669	0.675	0.604	1.079	0.605	0.83	1.072	1.123
16	0.663	0.725	0.652	0.66	0.619	0.954	1.092	0.961	0.999	1.179
17	0.671	0.689	0.675	0.699	0.68	1.008	0.796	1.03	0.932	0.879
18	0.708	0.729	0.763	0.603	0.569	1.052	1.004	0.95	0.952	0.962
19	0.785	0.691	0.641	0.545	0.72	1.089	1.092	0.99	0.978	1.079
20	0.657	0.703	0.801	0.725	0.674	1.158	0.872	0.927	0.905	0.999
21	0.748	0.527	0.691	0.676	0.63	0.978	0.977	0.97	1.03	0.923
22	0.584	0.685	0.701	0.684	0.782	1.034	1.053	1.012	0.984	0.99
23	0.693	0.696	0.699	0.621	0.548	0.953	0.894	1	1.043	0.905
24	0.642	0.642	0.765	0.66	0.576	1.019	0.873	0.855	1.172	1.074
25	0.684	0.658	0.784	0.576	0.695	0.915	0.882	1.06	1.077	0.888
26	0.68	0.637	0.648	0.585	0.607	0.934	1.079	1.08	1.179	0.919
27	0.722	0.747	0.63	0.622	0.62	1.199	1.1	1.049	0.88	1.092
28	0.681	0.734	0.688	0.645	0.54	1.009	1.075	0.706	0.984	0.972
29	0.659	0.565	0.649	0.62	0.593	0.868	1.002	1.067	1.043	0.973
30	0.602	0.675	0.624	0.69	0.709	0.889	0.959	0.969	1.135	0.987
31	0.68	0.619	0.655	0.641	0.618	0.903	0.939	1.16	1.128	1.026
32	0.686	0.623	0.609	0.739	0.598	1.134	0.969	1.015	1.106	0.973
33	0.565	0.572	0.525	0.693	0.721	1.12	0.903	0.93	1.002	1.135
34	0.65	0.607	0.553	0.609	0.667	1.076	1.028	0.958	1.026	0.993
35	0.42	0.668	0.676	0.49	0.597	0.755	0.965	1.086	1.052	1.084
36			0.613	0.679	0.64				1.067	1.124

Weight of birds in 2 mg/kg treatment group (A), 5 mg/kg treatment group (B), 10 mg/kg treatment group (C), negative control (D) and positive control (E) at day 35 (left) and day 42 (right).

	A	B	C	D	E	A	B	C	D	E
1	1.482	1.326	1.515	1.619	1.345	2.438	2.001	2.13	2.007	2.17
2	1.347	1.533	1.515	1.383	1.429	2.101	1.96	2.017	1.998	2.087
3	1.498	1.423	1.438	1.491	1.557	2.087	1.959	1.99	1.992	2.071
4	1.371	1.31	1.09	1.516	1.306	2.041	1.95	1.986	1.967	2.056
5	1.509	1.419	1.399	1.458	1.384	2.039	1.939	1.968	1.948	1.999
6	1.436	1.432	1.42	1.599	1.251	2.018	1.89	1.948	1.938	1.998
7	1.439	1.541	1.446	1.453	1.515	2.006	1.876	1.943	1.928	1.989
8	1.231	1.325	1.325	1.439	1.431	2.002	1.866	1.907	1.927	1.982
9	1.61	1.451	1.378	1.586	1.609	1.997	1.846	1.902	1.922	1.974
10	1.554	1.578	1.599	1.43	1.381	1.978	1.844	1.9	1.902	1.973
11	1.474	1.503	1.286	1.436	1.553	1.929	1.84	1.885	1.895	1.969
12	1.292	1.326	1.36	1.237	1.328	1.908	1.839	1.876	1.886	1.947
13	1.106	1.412	1.156	1.566	1.531	1.907	1.811	1.858	1.885	1.902
14	1.408	1.305	1.47	1.427	1.312	1.901	1.801	1.832	1.885	1.889
15	1.263	1.277	1.429	1.46	1.277	1.895	1.797	1.831	1.875	1.855
16	1.376	1.235	1.365	1.475	0.958	1.876	1.769	1.812	1.868	1.839
17	1.385	1.496	1.501	1.506	1.362	1.858	1.754	1.81	1.864	1.836
18	1.424	1.264	1.35	1.442	1.362	1.839	1.748	1.805	1.852	1.829
19	1.543	1.491	1.235	1.542	1.442	1.825	1.706	1.798	1.843	1.815
20	1.403	1.285	1.428	1.526	1.455	1.825	1.705	1.769	1.826	1.809
21	1.358	0.993	1.519	1.302	1.635	1.795	1.704	1.763	1.817	1.806
22	1.472	1.271	1.268	1.54	1.504	1.794	1.698	1.752	1.817	1.805
23	1.457	1.296	1.399	1.535	1.654	1.79	1.68	1.733	1.809	1.801
24	1.378	1.489	1.248	1.447	1.487	1.775	1.64	1.725	1.808	1.792
25	1.413	1.331	1.454	1.603	1.309	1.765	1.629	1.723	1.807	1.79
26	1.608	0.906	1.442	1.542	1.348	1.748	1.625	1.693	1.802	1.786
27	1.586	1.215	1.356	1.512	1.383	1.699	1.619	1.69	1.791	1.785
28	1.358	1.416	1.332	1.396	1.385	1.615	1.605	1.671	1.786	1.752
29	1.628	1.568	1.529	1.52	1.583	1.598	1.598	1.667	1.782	1.752
30	1.506	1.177	1.172	1.442	1.209	1.591	1.588	1.639	1.755	1.752
31	0.941	1.298	1.379	1.521	1.549	1.585	1.584	1.614	1.749	1.726
32	1.352	1.403	1.256	1.525	1.364	1.575	1.58	1.599	1.733	1.709
33	1.326	1.462	1.37	1.685	1.507	1.501	1.526	1.545	1.709	1.676
34	1.286	1.243	1.271	1.601	1.351	1.28	1.213	1.543	1.671	1.64
35	0.825	1.533	1.725	1.733	1.644	1.203	1.208	1.453	1.65	1.609
36			1.495	1.347	1.415			1.399	1.562	1.368

Feed weighed in (in) and weighed out (out) in Kg for 2 mg/kg treatment group (Group A), 5 mg/kg treatment group (Group B), 10 mg/kg treatment group (Group C), negative control (Group D) and positive control (Group E) for the 21 day dosing period.

	Group A		Group B		Group C		Group D		Group E	
	out	in	out	in	out	in	out	in	out	in
Day 21										
16/11/2004										
17/11/2004										
18/11/2004		10		10		10		10		10
19/11/2004										
20/11/2004										
21/11/2004		3.89		3.844		3.832		3.561		2.875
22/11/2004	1.65	8.35	2.103	7.897	0.902	10	0.244	9.756	0.62	9.38
Day 28										
23/11/2004										
24/11/2004		5		5		5		5		5
25/11/2004		5.5		5.5		5.5		5.5		5.5
26/11/2004		5.575		5.069		4.34		5.57		4.945
27/11/2004		7		8		7		7		7
28/11/2004										
29/11/2004	4.883	5.5	4.964	5.5	3.294	5.5	2.276	5.5	2.514	5.5
Day 35										
30/11/2004		6.16		7		7		7		7
01/12/2004										
02/12/2004		9		9		9		9		9
03/12/2004		5.5		5.5		5.5		5.5		5.5
04/12/2004										
05/12/2004		5.5		5.5		5.5		5.5		5.5
06/12/2004	4.184		5.824		2.948		3.686		1.268	