# IMPROVEMENT OF CHEMICAL ANALYSIS OF ANTIBIOTICS

## VIII*. APPLICATION OF PREPACKED $C_{18}$ CARTRIDGE FOR THE ANALYSIS OF TETRACYCLINE RESIDUES IN ANIMAL LIVER

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SUMMARY

   A simple, rapid and precise analytical method for tetracycline (TC) residues in the liver of slaughtered animals has been established. The recoveries of oxytetracycline (OTC), TC, chlortetracycline (CTC) and doxycycline (DC) from beef liver spiked at the level of 1.0 ppm were 87.7, 87.5, 79.6 and 67.5% with coefficients of variations of 1.01–2.87%. Detection limits in beef liver were 0.05 and 0.1 ppm for OTC and TC and for CTC and DC, respectively. It is also possible to apply this method to the analysis of residual TCs in various foods with the same recovery, accuracy and detection limits as in the case of beef liver.

INTRODUCTION

   The tetracycline antibiotics (TCs) are widely used in modern agricultural practice. Oxytetracycline (OTC), tetracycline (TC), chlortetracycline (CTC) and doxycycline (DC) are most frequently applied in veterinary medicine and animal nutrition. In Japan, more than 60% of all antibiotics used for animals are TCs[1], and residual TCs have been found in the organs and muscles of slaughtered animals[2,3]. Therefore, the monitoring of such residues in slaughtered animals is one of the most important duties for a public health agency. Since the concentration of residual TCs in liver and kidney is 4–6 times that in muscle[4], inspection of the liver of slaughtered animals for residual TCs is a most effective means of monitoring safety of foods.
   Bioassays are most often used for the measurement of residual TCs in liver, but their precision appears to be variable, moreover, the specifity is questionable. Therefore, some chemical methods have been reported[2,5,6], however, most of them are complicated or insensitive and they cannot be successfully applied to routine residual analysis of TCs in liver.

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   * For Part VII, see *J. Chromatogr.*, 314 (1984) 303.

In the previous reports[7,8] we have established chemical methods for the determination of TCs in some foods using a combination of Sep-Pak $C_{18}$ clean-up and high-performance thin-layer chromatography (HPTLC) together with densitometry or visualization with suitable reagents. Although this method is simple, rapid and reliable, it did not yield satisfactory results for residual analysis of TCs in animal liver; furthermore, HPTLC is not always sensitive enough to allow detection of small amounts of TCs with good precision. However, these problems can be overcome by careful consideration of the following: (1) the difference in retention behaviour of TCs on $C_{18}$ cartridges from different suppliers; (2) the method of detection of TCs; (3) the elution of TCs from the $C_{18}$ cartridges; (4) the extraction of TCs from liver. Investigation of the above points had led to a simpler, more rapid and more precise analytical method for residual TCs in the liver of slaughtered animals.

In this paper, a technique for the determination of residual TCs in animal liver by a combination of clean-up on a prepacked $C_{18}$ cartridge (Baker 10 $C_{18}$) and the previously established high-performance liquid chromatography (HPLC)[9] is described, and applied to various foods.

EXPERIMENTAL

*Materials*

Metaphosphoric acid, perchloric acid, trichloroacetic acid, hydrochloric acid, methanol, acetonitrile, ethanol, oxalic acid, disodium ethylenediaminetetraacetate ($Na_2EDTA$), aqueous ammonia, citric acid and disodium hydrogen phosphate were analytical reagent grade materials.

OTC, TC, CTC and DC, as their hydrochlorides, were supplied by Pfizer Taito.

Sep-Pak $C_{18}$, Bond Elut $C_{18}$ (No. 607303) and Baker 10 $C_{18}$ (No. 7020-3) were purchased from Waters Assoc. (Milford, MA, U.S.A.), Analytichem International (Harbor City, CA, U.S.A.) and J. T. Baker (Phillipsburg, NJ, U.S.A.), respectively.

*Preparation of standard tetracycline solutions*

Each tetracycline (100 mg) was weighed accurately into a 10-ml volumetric flask and diluted to volume in methanol or water. Dilution was sometimes necessary.

*Extraction and clean-up procedure*

A sample (5 g) was blended three times with 20, 20 and 10 ml of 0.1 $M$ $Na_2EDTA$–McIlvaine buffer (pH 4.0) using a high-speed blender, and centrifuged at 700 $g$. After filtration of the supernatant, the filtrate was applied on a Baker 10 $C_{18}$ cartridge activated with methanol and water, the cartridge was washed with 20 ml of water. The TCs were eluted with 10 ml of 0.01 $M$ methanolic oxalic acid solution and collected in a 10-ml volumetric flassk.

*High-performance liquid chromatography*

A high-performance liquid chromatograph equipped with a constant-flow pump (Shimadzu LC-5A, Kyoto, Japan) was used, together with a variable-wavelength UV detector (Shimadzu SPD-2AM, Kyoto, Japan) operated at 350 nm. The separation was performed on LiChrosorb RP-8 (10 $\mu$m, 250 × 4.0 mm I.D., E.

Merck) with methanol–acetonitrile–0.01 $M$ aqueous oxalic acid solution, pH 2.0 (1:1.5:2.5) as the mobile phase at a flow-rate of 2 ml/min at room temperature. For the determination of TCs, each sample and the standard solution (100 $\mu$l) are injected.

## RESULTS AND DISCUSSION

### Adsorption of tetracyclines on the $C_{18}$ cartridges

When the TCs from animal liver (20 g) were examined according to the previous Sep-Pak $C_{18}$ clean-up system[7], their recoveries decreased remarkably. In order to determine the influence of liver weight on the recoveries of TCs, decreasing amounts of liver were employed at the level of 0.1 ppm. It was impossible to use our HPTLC method, because this was not precise enough to allow the determination of small amounts of TCs[7]. However, the previously established HPLC system[9] was suitable. Accordingly, after addition of TCs (1 $\mu$g/g) to the extract they were determined in accordance with the previous Sep-Pak $C_{18}$ clean-up followed by HPLC, and their recoveries were calculated. As shown in Fig. 1, the recoveries improve with decreasing liver weight, indicating that impurities from liver undoubtedly interfere with the adsorption of TCs on Sep-Pak $C_{18}$. When TCs were added to an extract from 5 g of beef liver, the recoveries were 95.0, 93.6, 85.6 and 74.3% for OTC, TC, CTC and DC, respectively. Although these recoveries do not include the loss from the extraction step, their values are better than those obtained in the previous experiment.
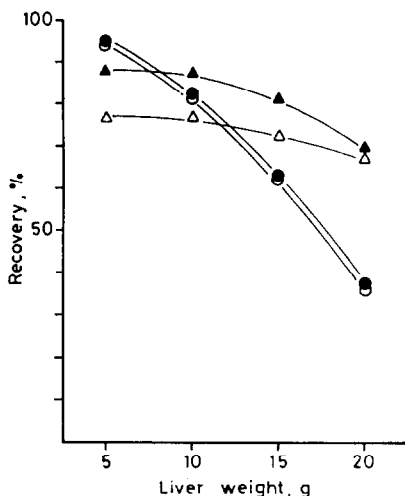


Fig. 1. Influence of animal liver weight on the recoveries of TCs. Recoveries of TCs fortified to the extract from beef liver at the level of 1.0 ppm using previous Sep-Pak $C_{18}$ clean-up. Results of three replicates. ●, OTC; ○, TC; ▲, CTC; △, DC.

We have reported that Sep-Pak $C_{18}$ cartridges can be successfully applied to the analysis of TCs in fish tissues, whereas Bond Elut $C_{18}$ cartridge cannot[7]. This result suggests that a large difference in retention behaviour of TCs occurs on $C_{18}$ cartridges from different suppliers. If a $C_{18}$ cartridge with adsorbing power stronger than that of Sep-Pak $C_{18}$ is used for the analysis of TCs, more satisfactory results

can be expected. In order to clarify the difference in adsorbing power for TCs among Sep-Pak $C_{18}$, Bond Elut $C_{18}$ and Baker 10 $C_{18}$, the following experiment was carried out with TC as a typical sample: aqueous solutions (10 ml) containing various amounts of TC (5–70 mg) were applied on the $C_{18}$ cartridges and then unretained TC was determined by HPLC. The difference in adsorbing power for TC among these cartridges is clear from Table I. The retention of TC on Sep-Pak $C_{18}$ is weaker than on the others. Consequently, the subsequent experiments were carried out using Bond Elut $C_{18}$ and Baker 10 $C_{18}$.

TABLE I

COMPARISON OF RETENTION BEHAVIOUR OF TCs ON $C_{18}$ CARTRIDGES

Amount of TC unretained on the $C_{18}$ cartridges on applying 10 ml of aqueous TC solution. Results of three replicates.

| TC (mg) | TC unretained (mg) | | |
|---|---|---|---|
| | Sep-Pak $C_{18}$ | Baker 10 $C_{18}$ | Bond Elut $C_{18}$ |
| 5 | 0 | 0 | 0 |
| 10 | 0 | 0 | 0 |
| 15 | 0 | 0 | 0 |
| 20 | 0 | 0 | 0 |
| 30 | 0 | 0 | 0 |
| 40 | 0.2 | 0 | 0 |
| 50 | 0.6 | 0 | 0 |
| 60 | 12.6 | 11.3 | 3.6 |
| 70 | 18.8 | 17.8 | 8.8 |

*Elution of tetracyclines from the $C_{18}$ cartridges*

TCs form chelate complexes with metal ions[10,11] and are capable of adsorption on alkyl-bonded reversed-phase (RP) columns[12], so that they are not eluted from $C_{18}$ cartridges with methanol, acetonitrile or ethanol. In the case of the Sep-Pak $C_{18}$ clean-up system[7], pretreatment of the $C_{18}$ cartridge with 0.2 $M$ aqueous $Na_2EDTA$ solution made possible the elution of TCs from the cartridge with ethanol, however, this pretreatment was ineffective for Bond Elut $C_{18}$ and Baker 10 $C_{18}$. We had already found that only a mobile phase containing oxalic acid gave excellent separation of TCs by RP-TLC[13] and RP-HPLC[9]. Since the separations using a $C_{18}$ cartridge and a RP column are based on the same principle, we supposed that TCs can be eluted from $C_{18}$ cartridges with methanol containing oxalic acid, and the required concentration and volume of methanolic oxalic solution were examined. After retention of TCs (each 5 $\mu$g) on Bond Elut $C_{18}$ and Baker 10 $C_{18}$, they were eluted using 10 ml of methanol containing various amounts of oxalic acid and were then determined. All TCs were completely recovered from both cartridges using > 0.01 $M$ methanolic oxalic acid solution, and typical elution curves of TC are shown in Fig. 2. When various volumes of 0.01 $M$ methanolic oxalic acid solution were used as eluent in the same manner as in the above experiment, good recoveries of all TCs were obtained above 7 ml and 5 ml from Bond Elut $C_{18}$ and Baker 10 $C_{18}$, respec-
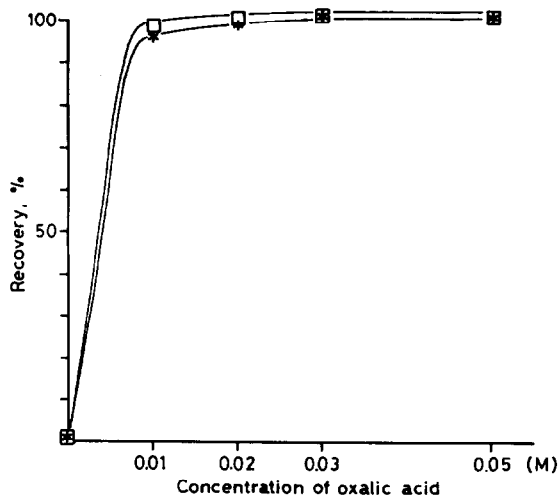
Fig. 2. Effect of oxalic acid concentration on the recoveries of TCs from $C_{18}$ cartridges using 10 ml of methanolic oxalic acid solution. Results of three replicates. $\square$, Baker 10 $C_{18}$; $*$, Bond Elut $C_{18}$.

tively (Fig. 3). Because it is more convenient and efficient to use the same volume for both cartridges, 10 ml of 0.0 $M$ methanolic oxalic acid solution were employed in subsequent work.
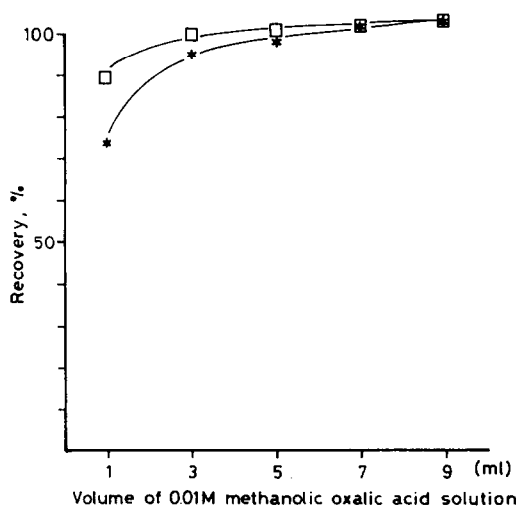


Fig. 3. Effect of volume of 0.01 $M$ methanolic oxalic acid solution on recovery of TC from $C_{18}$ cartridges. Results of three replicates. Cartridges as in Fig. 2.

*Application of solvents to the $C_{18}$ cartridges*

As TCs are apt to bind to proteins in biological samples[14,15], it has been considered that strong acids[6,16,17] and acidic deproteinizing agents[2,15,18,19] are suitable for the extraction of TCs from biological samples. Therefore, the suitability of addition of various solvents to $C_{18}$ cartridges was investigated. Using 60 ml of var-

ious deproteinizing agents (5% aqueous trichloroacetic acid, 5% aqueous perchloric acid, 5% aqueous phenol and 1% aqueous metaphosphoric acid solution), 1 $M$ aqueous hydrochloric acid solution, McIlvaine buffer (pH 4.0) and 0.1 $M$ $Na_2EDTA$–McIlvaine buffer (pH 4.0), TCs (each 5 $\mu$g) were applied on Bond Elut $C_{18}$ or Baker 10 $C_{18}$ and then eluted with 10 ml of 0.01 $M$ methanolic oxalic acid solution and determined. Table II indicates that satisfactory results are obtained by the use of McIlvaine buffer (pH 4.0), 0.1 $M$ $Na_2EDTA$–McIlvaine buffer (pH 4.0), 5% aqueous trichloroacetic acid solution and 1% aqueous metaphosphoric acid solution. It was considered that these results are due to the instability of TCs and the packing materials of the cartridges in the strongly acidic medium[20]. The suitability of extractants will be investigated in future experiments.

TABLE II

APPLICATION OF SOLVENTS TO THE $C_{18}$ CARTRIDGES

Recovery (%) from 60 ml solvents containing TCs (each 5 $\mu$g). Results of three replicates.

| Solvent | Bond Elut $C_{18}$ | | | | Baker 10 $C_{18}$ | | | |
|---|---|---|---|---|---|---|---|---|
| | OTC | TC | CTC | DC | OTC | TC | CTC | DC |
| 0.1 $M$ $Na_2EDTA$– McIlvaine buffer (pH 4.0) | 96 | 96 | 96 | 98 | 100 | 100 | 98 | 98 |
| McIlvaine buffer (pH 4.0) | 98 | 106 | 98 | 93 | 97 | 100 | 100 | 94 |
| 5% Metaphosphoric acid (aq.) | 100 | 100 | 99 | 95 | 96 | 89 | 90 | 87 |
| 5% Trichloroacetic acid (aq.) | 94 | 95 | 94 | 92 | 92 | 83 | 99 | 90 |
| 5% Phenol (aq.) | 63 | 69 | 75 | 64 | 35 | 74 | 82 | 77 |
| 1 $M$ Hydrochloric acid (aq.) | 63 | 56 | 60 | 55 | 58 | 44 | 62 | 53 |
| 5% Perchloric acid (aq.) | 71 | 62 | 66 | 68 | 57 | 42 | 57 | 52 |

*Analysis of tetracyclines in beef liver*

In order to apply the above results to the analysis of TCs in animal liver, we first tested whether or not TCs were retained on the $C_{18}$ cartridges in the impurities from liver. The extract (prepared from 5 g of beef liver using 0.1 $M$ $Na_2EDTA$– McIlvaine buffer) containing TCs (each 5 $\mu$g) was applied on Bond Elut $C_{18}$ or Baker 10 $C_{18}$ and then the eluted by 10 ml of 0.01 $M$ methanolic oxalic acid solution. Although the determination of TCs was attempted, interfering peaks appeared in the chromatograms and the precise determination of TCs was impossible. The interfering substances were almost eliminated by washing the $C_{18}$ cartridges with 20 ml of water after application of the extracts, and the recoveries of all TCs from both $C_{18}$ cartridges were very close to 100%. These results show that the both cartridges adsorb TCs in large amounts of impurities from liver and the procedure enables the precise determination of TCs. Because the operation with Baker 10 $C_{18}$ was simpler and more rapid than that with Bond Elut $C_{18}$, we chose Baker 10 $C_{18}$ as the cartridge for clean-up in subsequent experiments.

In order to investigate the suitability of various extractants for the analysis of TCs in liver, TCs from beef liver fortified at the level of 1.0 ppm were extracted in three steps using 20, 10 and 10 ml of the extractants (0.1 $M$ $Na_2EDTA$–McIlvaine

buffer, McIlvaine buffer, 5% aqueous trichloroacetic acid solution and 1% aqueous metaphosphoric acid solution). Table III shows that 0.1 $M$ Na$_2$EDTA–McIlvaine buffer and McIlvaine buffer are most suitable. It has been reported that the presence of EDTA improves the recoveries of TCs from liver homogenate[15], so 0.1 $M$ Na$_2$EDTA–McIlvaine buffer (pH 4.0) was selected as extractant in the following work.

TABLE III

COMPARISON OF EXTRACTANTS

Recovery of TCs from 5 g of homogenized beef liver fortified at the level of 1.0 ppm using three-step extractions (20, 10 and 10 ml). Results of three replicates.

| Extractant | Recovery (%) | | | |
|---|---|---|---|---|
| | OTC | TC | CTC | DC |
| 0.1 $M$ Na$_2$EDTA–McIlvaine buffer (pH 4.0) | 87 | 85 | 75 | 59 |
| McIlvaine buffer (pH 4.0) | 88 | 84 | 75 | 59 |
| 5% Trichloroacetic acid (aq.) | 76 | 77 | 52 | 35 |
| 5% Metaphosphoric acid (aq.) | 73 | 75 | 64 | 47 |

However, since the recovery is not always adequate, especially for DC even using 0.1 $M$ Na$_2$EDTA–McIlvaine buffer, a more effective extraction was sought. As shown in Table IV, it is most effective to extract in three sterps with 20, 20 and 10 ml, and the recoveries are much better than those obtained by Ryan and Dupont[6], Terada et al.[2] and our previous examination. Consequently, it is concluded that the most suitable analytical method for TC residues in animal liver is as follows: animal liver (5 g) is blended three times with 20, 20 and 10 ml of 0.1 $M$ Na$_2$EDTA–McIlvaine buffer (pH 4.0) and centrifuged. The supernatant is applied on a Baker 10 C$_{18}$ cartridge, the TCs are eluted with 10 ml of 0.01 $M$ methanolic oxalic acid solution and are determined by HPLC.

TABLE IV

EFFICIENCY OF EXTRACTION UNDER VARIOUS CONDITIONS

Recovery of TCs from 5 g of homogenized beef liver fortified at the level of 1.0 ppm, using 0.1 $M$ Na$_2$EDTA–McIlvaine buffer as extractant. Results of three replicates.

| Volumes (ml) | Recovery (%) | | | |
|---|---|---|---|---|
| | OTC | TC | CTC | DC |
| 20 | 64 | 64 | 51 | 36 |
| 20, 10 | 76 | 77 | 64 | 52 |
| 20, 10, 10 | 87 | 85 | 75 | 59 |
| 20, 20 | 79 | 76 | 71 | 52 |
| 20, 20, 10 | 87 | 87 | 80 | 68 |
| 20, 20, 20 | 87 | 84 | 80 | 68 |

*Application to various foods*

We consider that the established analytical method for TCs in beef liver can be applied to the analysis of TCs in various foods. So TCs in various foods spiked at the level of 1.0 ppm were determined according to the Experimental and then the recoveries were calculated. As shown in Table V and Fig. 4, good recoveries and coefficients of variation (C.V.) were obtained with no interfering peaks and the detection limits were 0.05 and 0.1 ppm for OTC and TC and for CTC and DC, respectively. The present method enables the analysis of TCs in various foods with good recoveries (67.5–94.9%), high accuracy (C.V. 0.59–3.50%) and good detection limits. Further, it is very simple and rapid (only 1.5 h for four samples). Therefore, we recommend the method for the residual analysis of TCs in various foods.
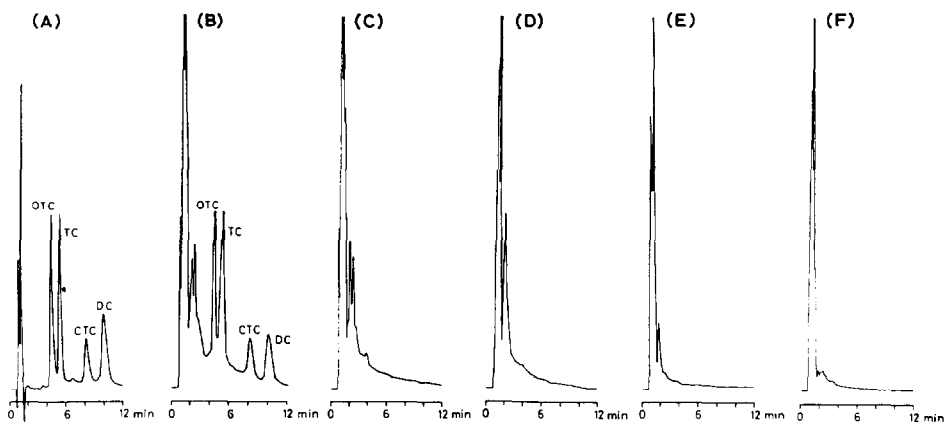


Fig. 4. Typical high-performance liquid chromatograms of various food extracts. A, Standard of TCs (50 ng); B, fortified (1 ppm) beef liver extract; C, beef liver extract; D, beef kidney extract; E, eel extract; F, milk extract.

TABLE V

RECOVERY OF TCs FROM FORTIFIED FOODS

Homogenized samples prepared from 5 g of various foods fortified at the level of 1.0 ppm.

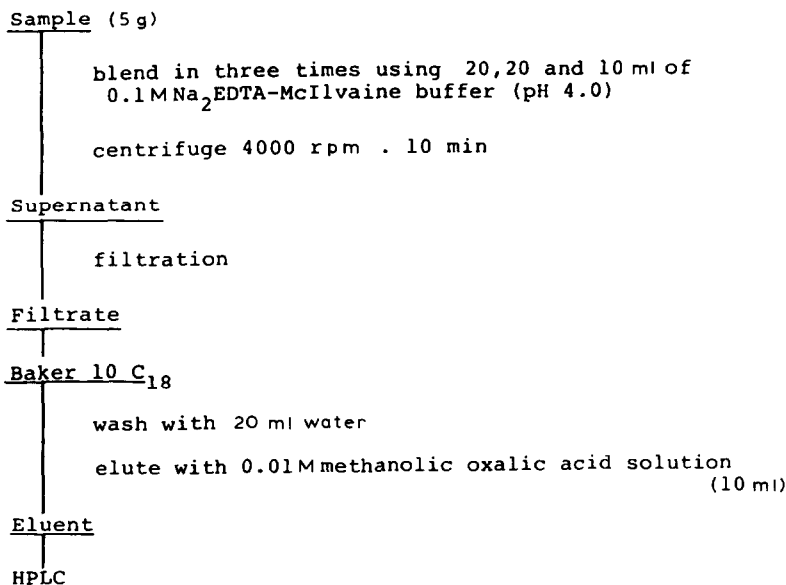| Sample | No. of samples | Recovery (%) (C.V., %) | | | |
| --- | --- | --- | --- | --- | --- |
| | | OTC | TC | CTC | DC |
| Beef liver | 4 | 87.7 (2.37) | 87.5 (1.01) | 79.6 (2.87) | 67.5 (2.00) |
| Pork liver | 4 | 91.0 (1.14) | 88.1 (1.64) | 88.5 (2.55) | 68.9 (3.33) |
| Chicken liver | 5 | 85.6 (1.91) | 81.4 (1.67) | 80.8 (1.99) | 70.3 (0.72) |
| Beef kidney | 5 | 90.7 (2.69) | 85.9 (1.03) | 92.3 (2.62) | 81.4 (2.86) |
| Pork kidney | 5 | 89.1 (1.19) | 86.3 (2.33) | 86.2 (2.13) | 83.2 (2.20) |
| Beef muscle | 5 | 91.6 (2.50) | 87.5 (2.70) | 93.0 (2.07) | 85.4 (3.52) |
| Pork muscle | 5 | 92.8 (1.42) | 90.2 (1.64) | 94.9 (1.39) | 85.5 (2.22) |
| Chicken muscle | 5 | 91.3 (0.59) | 90.0 (0.71) | 92.6 (1.33) | 84.2 (0.44) |
| Milk | 5 | 86.8 (0.82) | 83.4 (0.90) | 86.5 (0.59) | 84.2 (1.41) |
| Egg | 4 | 81.6 (0.74) | 80.0 (0.78) | 82.3 (1.57) | 85.5 (2.05) |
| Yellow tail | 5 | 91.0 (3.50) | 87.8 (2.47) | 93.4 (2.91) | 83.4 (0.93) |
| Eel | 5 | 93.6 (1.07) | 88.9 (0.83) | 87.5 (3.06) | 85.2 (1.44) |

Sample (5 g)

    blend in three times using 20,20 and 10 ml of
    0.1 M $Na_2$EDTA-McIlvaine buffer (pH 4.0)

    centrifuge 4000 rpm . 10 min

Supernatant

    filtration

Filtrate

Baker 10 $C_{18}$

    wash with 20 ml water

    elute with 0.01 M methanolic oxalic acid solution
                                        (10 ml)

Eluent

HPLC

Fig. 5. Analytical procedure for TCs in animal liver.

CONCLUSION

A simple and rapid technique for the analysis of TC residues in animal liver has been established. The analytical procedure is summarized in Fig. 5. The recoveries of OTC, TC, CTC and DC from beef liver spiked at the level of 1.0 ppm are 87.7, 87.5, 79.6 and 67.5% with good coefficients of variation (1.01–2.87%), respectively. The detection limits in beef liver are 0.05 and 0.1 ppm for OTC and TC, and for CTC and DC, respectively. It is also possible to apply this method to the analysis of residual TCs in various foods with the same recovery, accuracy and detection limits as in the case of beef liver.

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