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## Research Note

# Screening Procedures for Clenbuterol Residue Determination in Raw Swine Livers Using Lateral-Flow Assay and Enzyme-Linked Immunosorbent Assay

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## ABSTRACT

Clenbuterol, which may cause symptoms of increased heart rate, muscular tremors, headache, nausea, and muscular cramps in patients, has been prohibited for consumption in many countries including the European Union, the United States, and China. A rapid lateral-flow strip assay was developed in our laboratory, and results obtained with this assay were compared with those obtained with a commercial enzyme-linked immunosorbent assay (ELISA) kit for the screening of clenbuterol in raw swine liver. A total of 128 swine livers were acquired from five local markets and prepared for analysis by the lateral-flow strip assay and ELISA. Analysis was completed in 10 min with the lateral-flow strip assay and in 90 min with the ELISA. In parallel with the ELISA, the rapid detection strip produced no false-negative results but had a false-positive rate of 6.3%. Cross-reactivity of the strip was assessed and was negative after tests with clenbuterol analogues such as terbutaline, salbutamol, ractopamine, ritodrine, and fenoterol. These data suggest that a lateral-flow strip assay can be used safely as a screening method as part of a clenbuterol residue surveillance program and should be a valuable tool in the food safety field, especially in developing countries.

Clenbuterol (4-amino-(*t*-butylamino) methyl)-3,5-dichloro benzylalcohol hydrochloride) belongs to the family of β-agonists, which currently are used as bronchodilators for the treatment of asthma in humans and as tocolytic agents in veterinary medicine. However, clenbuterol has been used illegally in animals to promote growth of muscle mass and decrease fat accumulation. Proliferation of the illegal use of clenbuterol to promote animal growth raised new concerns about its safety and toxicity, particularly for consumers of these treated animals. Various types of intoxication have been described in some countries as due to the ingestion of liver and meat containing clenbuterol residues. An outbreak of illness in 1992 that affected 113 persons who consumed contaminated beef liver took place in Spain. Clenbuterol was detected in 47 urine samples in amounts ranging from 11 to 486 ppb *(19).* Victims were hospitalized with reversible symptoms of increased heart rate, muscle tremors, headache, nausea, fever, and chills. In August 1996, 62 persons asked for medical help in Italy. All patients had consumed beef 10 min to 3 h before symptoms developed. Concentrations of clenbuterol in the meat ranged from 0.8 to 7.4 mg/kg *(21).* A similar incident traced to beef liver involved 22 persons in France *(17).* Clenbuterol also is a concern in Portugal *(2)* and Hong Kong *(4).*

Many analytical methods for the identification of clenbuterol in different biological matrices have been described. The enzyme-linked immunosorbent assay (ELISA) *(15, 16, 20)* is the most sensitive detection system for this compound and has been used for screening. Quantification and confirmation usually have been accomplished with methods based on high-performance liquid chromatography *(6, 7, 27),* liquid chromatography coupled with mass spectrometry *(14),* and gas chromatography coupled with mass spectrometry *(1, 9, 10, 18, 22).*

Lateral-flow assays, which have been used as diagnostic tools for monitoring drugs *(25),* toxins *(23, 24, 26),* hormones *(8),* and pathogens *(3),* allow a rapid and qualitative identification of analytes. This technique is based on an immunochromatographic procedure that utilizes antigen-antibody properties in a novel way and provides rapid detection. The four major advantages of this method are (i) user-friendly format, (ii) very short period (10 min) to obtain test results, (iii) long-term stability over a wide range of climates, and (iv) relatively inexpensive. These characteristics make it ideally suited for on-site testing with untrained personnel. The method for screening clenbuterol in a urine–colloidal gold immunochromatographic assay was developed and certified by the Chinese Ministry of Agriculture (NY/T933-2005) in 2005 and has been used since then in China *(12).*

The persistence of clenbuterol in plasma and urine is low, but clenbuterol persists in the liver at much higher levels than in other edible tissues and is detectable in the

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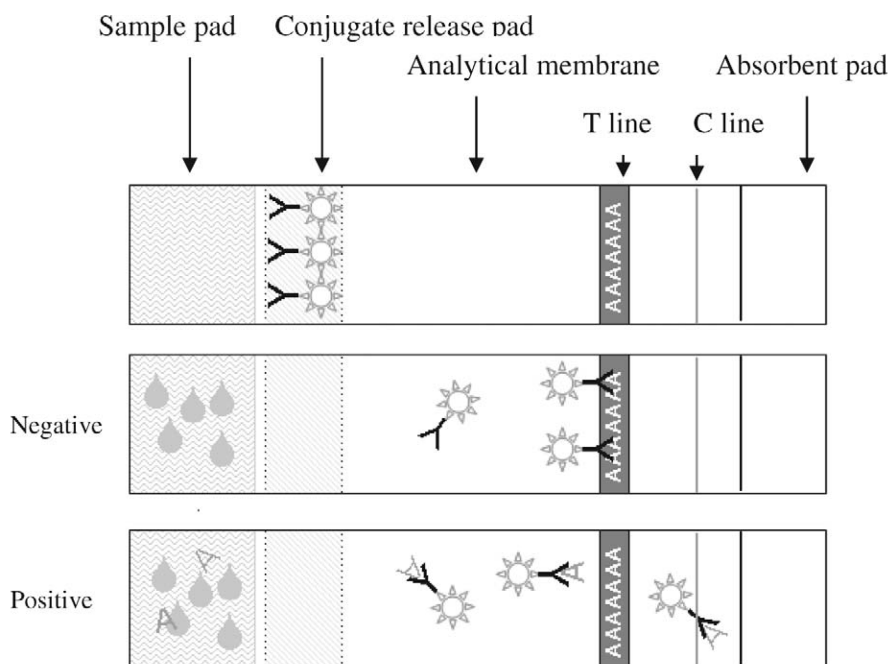


FIGURE 1. *Diagram of clenbuterol lateral-flow strip.*

liver for up to 2 weeks after the withdrawal of the drug from an animal's diet. Therefore, the liver is the tissue of choice for detecting illegal use of clenbuterol *(13).*

A rapid lateral-flow strip assay was developed in our laboratory, and the results obtained were compared with those obtained with a commercial ELISA kit for screening for clenbuterol in raw swine liver.

## MATERIALS AND METHODS

**Materials.** Terbutaline, clenbuterol, salbutamol, ritodrine, fenoterol, bovine serum albumin (BSA), and goat anti-mouse antibody were obtained from Sigma (St. Louis, Mo.). Ractopamine was obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Monoclonal antibody (R7) was produced in our laboratory by the Kohler and Milstein method *(11)* and was purified with a protein A affinity column (Pharmacia, Uppsala, Sweden). Hydrogen tetrachloroaurate trihydrate was obtained from Aldrich (Milwaukee, Wis.). The sample pad, conjugate release pad, analytical membrane, and absorbent pad were obtained from Schleicher & Schuell GmbH (Dassel, Germany). All solvents and other chemicals were analytical reagent grade. The XYZ Platform combining motion control with a BioJet Quanti3000k dispenser used to dispense clenbuterol-BSA or anti-mouse immunoglobulin (Ig) G to the nitrocellulose membrane and the AirJet Quanti3000k dispenser to dispense detector antibody onto the conjugate release pad were purchased from BioDot (Irvine, Calif.).

**Liver samples.** One hundred twenty-eight swine livers were acquired from five local markets in Nanchang, China, between December 2005 and January 2006. The livers were homogenized, accurately weighed (10 g wet mass), and stored at −20°C until used.

**ELISA kit.** The Ridascreen Clenbuterol Fast Kit (R1701, R-Biopharm, Darmstadt, Germany) contained a 96-well microtiter plate (12 strips with eight removable wells coated with antibodies directed against anti-clenbuterol antibodies), six clenbuterol standard solutions, peroxidase-conjugated clenbuterol, anti-clenbuterol antibody, substrate-chromogen solution, stop solution, and buffer. The detection limit of this assay for clenbuterol in livers is 40 ppt.

**Preparation of colloidal gold.** Five milliliters of a 1% (wt/vol) stock solution of hydrogen tetrachloroaurate trihydrate was added to 500 ml of distilled water and heated to boiling. Five milliliters of a freshly made 1% solution of sodium citrate was added to this gold solution under constant stirring, and the mixture was boiled until it turned red. After an additional 5 min of boiling, the solution was cooled to 4°C for further processing.

**Preparation of colloidal gold probe.** Colloidal gold was used for conjugation of IgG. Protein A gel-purified anti-clenbuterol monoclonal antibody (2 ml, 0.5 mg/ml, in 5 mM Tris-HCl, pH 7.5) was added to 20 ml of pH-adjusted colloidal gold solution and agitated for 30 min. Two milliliters of 1% (wt/vol) BSA solution was then added and agitated for 15 min. The mixture was centrifuged at 8,000 × *g* for 30 min, and the gold pellets obtained were dissolved in 50 mM Tris-HCl buffer.

**Preparation of immunochromatographic test strips.** The sample pad was treated with 50 mM borate buffer, pH 7.4, containing 1% BSA, 0.5% Tween 20, and 0.05% sodium azide and then dried at 60°C for 2 h. Clenbuterol-BSA (0.038 mg/ml) and goat anti-mouse antibody (1.123 mg/ml) were applied to the nitrocellulose membrane at the test and control lines, respectively, and dried at 35°C for 3 h. An absorption pad was used without treatment. The colloidal gold probe was applied to a treated glass fiber membrane and completely dried at 35°C for 3 h. The nitrocellulose membrane, absorption pad, glass fiber membrane, and pretreated sample pad were assembled as the lateral-flow strip (Fig. 1).

**Extraction of liver.** A 10-g sample of minced liver was homogenized with 30 ml of an ethyl acetate–isopropanol (7:3, vol/vol) mixture. The tube containing the homogenate was centrifuged at 5,000 × *g* for 20 min at 5°C, the supernatant was evaporated under vacuum, and the dry residue was dissolved in 10 ml of 0.01 M perchloric acid. The resulting mixture was transferred to another tube and centrifuged at 6,000 × *g* for 10 min at 5°C, the supernatant was transferred to a clean tube, the pH was adjusted

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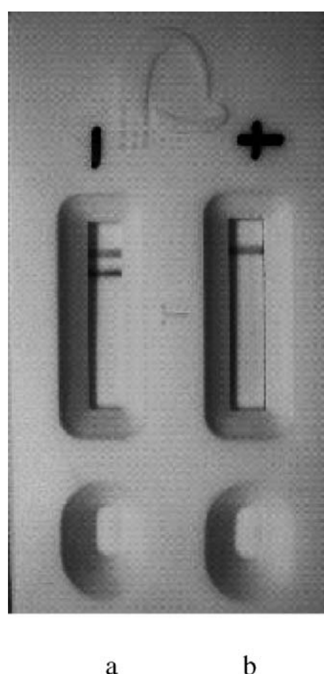

a b

FIGURE 2. *Diagram of a lateral-flow strip showing (a) a sample negative for clenbuterol and (b) a sample positive (1 ppb) for clenbuterol.*

to 8.5 with 2 M NaOH, and mixture was recentrifuged if a precipitate formed. The whole solution was passed through in an SPE column that had been washed with 5 ml of methanol and 5 ml of 0.05 M borate buffer (pH 8.5). After passage of the solution, the column was washed successively with 2 ml of water, 1 ml of 0.1 M acetate buffer (pH 4.0), and methanol. After drying under vacuum for 5 min, the analyte was eluted with dichloromethane–isopropanol–concentrated ammonia (80:20:2 by volume). The solution was evaporated under nitrogen, and the dry residue was dissolved in 10 ml of phosphate buffer (pH 7.4) for analysis by lateral-flow strip assay and ELISA.

**ELISA procedure.** Diluted antibody solution (100 μl) was added to the bottom of each assay well and incubated for 15 min at room temperature. After washing, 100 μl of diluted enzyme conjugate and 20 μl of standard or prepared samples were added to separate duplicate wells and incubated for 30 min at room temperature. After washing, 100 μl of substrate-chromogen solution was added and incubated for 15 min at room temperature in the dark. The reaction was stopped by adding 100 μl of stop solution. The absorbance was measured at 450 nm with a microplate reader (Labsystem, Helsinki, Finland).

**Assay of clenbuterol on the lateral-flow strip.** An 80-μl aliquot of sample was pipetted into the reaction holder, and 10 min was allowed for the antibody-antigen complex to form. One visual band (control line, which was close to the absorption pad) indicated a positive test result. Two visual bands (control line and test line) indicated a negative result (Fig. 2).

## RESULTS

**Clenbuterol residue in raw swine livers determined with the ELISA.** The ELISA results for detection of clenbuterol residue in raw liver are shown in Table 1. In China, the use clenbuterol in swine production is illegal. The maximum residue limit for clenbuterol has been set at 1 ppb.

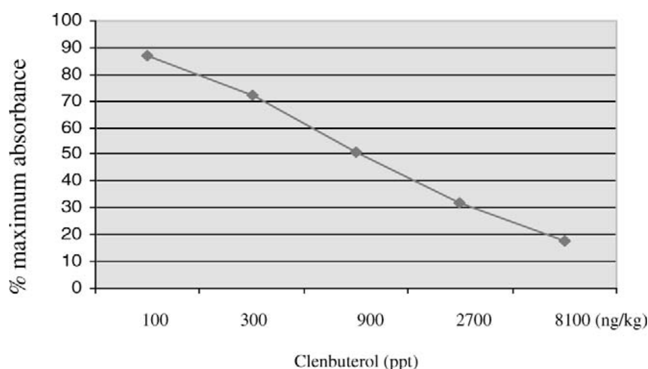


FIGURE 3. *Calibration curve of a Ridascreen Clenbuterol Fast Kit.*

According to this standard, 32 positive samples (≥1 ppb) and 96 negative samples (<1 ppb) were identified with the ELISA.

**Clenbuterol residue in raw swine livers determined with the lateral-flow strip assay.** All of the 32 ELISA-positive samples and 6 of the 96 ELISA-negative samples were positive for clenbuterol with the strip assay, and 90 of the 96 ELISA-negative samples were negative with the strip assay. Thus when compared with the ELISA, the lateral-flow strip assay had a false-positive rate of 6.3% and a false-negative rate of 0%. Overall relative accuracy obtained was 122 of 128 or 95.3%.

**Cross-reactivity of clenbuterol test strip.** Terbutaline, salbutamol, ritodrine, fenoterol, and ractopamine are most frequently associated with clenbuterol, and the cross-reactivity of clenbuterol test strips with these related β-agonists was examined at 1 to 100 ppb. Red bands were found in the test region. There was no cross-reactivity when clenbuterol lateral-flow strip was used to test these other β-agonist compounds.

## DISCUSSION

The lateral-flow strip test is a competitive binding immunoassay. The clenbuterol in the sample competes with the antigen on the nitrocellulose membrane for the limited binding sites of the antibody in the conjugate pad. When the clenbuterol level in the sample is below the cutoff level, the red conjugate will bind to the antigens coated on the nitrocellulose membrane, and a red test line will form, indicating a negative result. When clenbuterol is present in the sample at the cutoff level or higher, it will bind to antibodies in the conjugate pad and no red line will develop in the test region, indicating a positive result. The colored gold-antibody conjugate should bind to the control line and form a red band regardless of the presence of clenbuterol. With the ELISA, the mean absorbance values obtained for the standards and the samples are divided by the absorbance value of the first standard (zero standard) and then multiplied by 100. The zero standard is thus made equal to 100%, and the absorbance values are quoted in percentages. The values calculated for the standards are entered in a

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TABLE 1. *Detection of clenbuterol residue in raw swine liver with lateral-flow strip assay and ELISA*

| Sample no. | ELISA (ppb) | Lateral-flow strip assay | Sample no. | ELISA (ppb) | Lateral-flow strip assay | Sample no. | ELISA (ppb) | Lateral-flow strip assay |
|---|---|---|---|---|---|---|---|---|
| 1 | 10.6 | + | 44 | 0.2 | − | 87 | N | − |
| 2 | 21.5 | + | 45 | N | − | 88 | N | − |
| 3 | 27.6 | + | 46 | N | − | 89 | 0.2 | − |
| 4 | 50.9 | + | 47 | N | − | 90 | 0.2 | − |
| 5 | 27.4 | + | 48 | 0.1 | − | 91 | N | − |
| 6 | 21.3 | + | 49 | N | − | 92 | N | − |
| 7 | 34.0 | + | 50 | N | − | 93 | N | − |
| 8 | 15.6 | + | 51 | N | − | 94 | 0.1 | − |
| 9 | 13.5 | + | 52 | 0.2 | − | 95 | 0.9 | + |
| 10 | 0.5 | − | 53 | 19.3 | + | 96 | 16.4 | + |
| 11 | 11.3 | + | 54 | 18.7 | + | 97 | 23.6 | + |
| 12 | 0.3 | − | 55 | N | − | 98 | 21.6 | + |
| 13 | 0.4 | − | 56 | N | − | 99 | N | − |
| 14 | 0.2 | − | 57 | 0.2 | − | 100 | N | − |
| 15 | 0.2 | − | 58 | 16.5 | + | 101 | 0.2 | − |
| 16 | 0.1 | − | 59 | N | − | 102 | 31.0 | + |
| 17 | N[a] | − | 60 | N | − | 103 | 0.1 | − |
| 18 | 0.8 | + | 61 | 22.3 | + | 104 | 0.2 | − |
| 19 | 0.9 | + | 62 | 28.4 | + | 105 | N | − |
| 20 | N | − | 63 | N | − | 106 | 10.5 | + |
| 21 | N | − | 64 | 18.9 | + | 107 | N | − |
| 22 | 0.2 | − | 65 | 31.2 | + | 108 | N | − |
| 23 | N | − | 66 | 6.5 | + | 109 | N | − |
| 24 | 22.3 | + | 67 | 10.1 | + | 110 | 25.4 | + |
| 25 | 31.2 | + | 68 | N | − | 111 | N | − |
| 26 | 65.2 | + | 69 | N | − | 112 | N | − |
| 27 | 0.3 | − | 70 | N | − | 113 | 0.2 | − |
| 28 | 0.9 | + | 71 | 50.1 | + | 114 | 0.3 | − |
| 29 | N | − | 72 | 22.8 | + | 115 | 0.2 | − |
| 30 | 0.1 | − | 73 | 0.2 | − | 116 | N | − |
| 31 | 0.2 | − | 74 | 0.3 | − | 117 | N | − |
| 32 | N | − | 75 | N | − | 118 | N | − |
| 33 | 0.2 | − | 76 | N | − | 119 | N | − |
| 34 | 0.2 | − | 77 | 0.9 | + | 120 | N | − |
| 35 | 0.1 | − | 78 | N | − | 121 | N | − |
| 36 | N | − | 79 | N | − | 122 | N | − |
| 37 | N | − | 80 | N | − | 123 | N | − |
| 38 | 34.2 | + | 81 | N | − | 124 | N | − |
| 39 | 29.1 | + | 82 | 0.8 | + | 125 | N | − |
| 40 | N | − | 83 | 0.1 | − | 126 | 0.2 | − |
| 41 | 0.2 | − | 84 | 0.3 | − | 127 | 0.1 | − |
| 42 | 0.1 | − | 85 | N | − | 128 | 0.1 | − |
| 43 | 0.1 | − | 86 | N | − | | | |

[a] N, negative.

system of coordinates on semilogarithmic graph paper against the clenbuterol concentration in parts per billion. The calibration curve should be virtually linear in the 200 to 2,000 ppt range. The clenbuterol equivalents in parts per thousand corresponding to the absorbance of each sample can be read from the calibration curve (Fig. 3).

Liver, the alimentary matrix where clenbuterol is found in the highest concentrations and for the longest time, has been responsible for the largest number of clenbuterol intoxications *(5)*. In this study, 31 of 32 clenbuterol-positive samples had a high concentration of clenbuterol, i.e., more than 10 ppb.

The ELISA is the most sensitive detection system for clenbuterol and has been used for screening of various tissues for this compound. With both the ELISA and the lateral-flow strip assay, a confirmatory technique such as gas or liquid chromatography plus mass spectrometry is required because of possible cross-reactions and to avoid false-positive results. The advantages of the lateral-flow strip assay over the ELISA include rapid results, less sample processing required, inexpensive, yes-no answers generated without use of an instrument, and ease of use in the field by nonlaboratory personnel. In spite of the inability to accurately quantify the analyte, the strip assay should be

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a valuable tool in the food safety field, especially in developing countries.

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## REFERENCES

1. Amendola, L., C. Colamonici, F. Rossi, and F. Botre. 2002. Determination of clenbuterol in human urine by GC–MS–MS–MS: confirmation analysis in antidoping control. *J. Chromatogr. B* 773:7–16.
2. Barbosa, J., C. Cruz, J. Martins, J. M. Silva, C. Neves, C. Alves, F. Ramos, and M. L. Da Silveira. 2005. Food poisoning by clenbuterol in Portugal. *Food Addit. Contam.* 22:563–566.
3. Brandonisio, O., L. Fumarola, P. Maggi, R. Cavaliere, R. Spinelli, and G. Pastore. 2002. Evaluation of a rapid immunochromatographic test for serodiagnosis of visceral leishmaniasis. *Eur. J. Clin. Microbiol. Infect. Dis.* 21:461–464.
4. Chan, T. Y. K. 1998. Food poisoning following the consumption of clenbuterol-treated livestock. *Hong Kong Pract.* 20:366–369.
5. Cristino, A., F. Ramos, and M. I. N. Silveira. 2003. Control of the illegal use of clenbuterol in bovine production. *J. Pharm. Biomed. Anal.* 32:311–316.
6. Gianfranco, B., F. Maurizio, R. Barbara, and C. Vittorio. 2001. Use of molecularly imprinted polymers in the solid-phase extraction of clenbuterol from animal feeds and biological matrices. *J. Chromatogr. B* 759:27–32.
7. Grzegorz, B., and J. N. Luc. 2003. Simultaneous high-throughput determination of clenbuterol, ambroxol and bromhexine in pharmaceutical formulations by HPLC with potentiometric detection. *J. Pharm. Biomed. Anal.* 32:887–903.
8. Henderson, K., and J. Stewart. 2000. A dipstick immunoassay to rapidly measure serum oestrone sulfate concentrations in horses. *Reprod. Fertil. Dev.* 12:183–189.
9. Hernández-Carrasquilla, M. 2000. Gas chromatography–mass spectrometry analysis of β-agonists in bovine retina. *Anal. Chim. Acta* 408:285–290.
10. Keskin, S., D. Ozer, and A. Temizer. 1998. Gas chromatography–mass spectrometric analysis of clenbuterol from urine. *J. Pharm. Biomed. Anal.* 18:639–644.
11. Kohler, G., and C. Milstein. 1975. Continuous culture of fused cells secreting antibody of predefined specificity. *Nature* 256:495–497.
12. Lai, W. H., Y. Xu, D.Y. C. Fung, and Y. H. Xiong. 2007. Development of a lateral-flow assay for rapid screening of the performance-enhancing sympathomimetic drug clenbuterol used in animal production; food safety assessments. *Asia Pac. J. Clin. Nutr.* 16(Suppl. 1):106–110.
13. Meyer, H. H., and L. M. Rinke. 1991. The pharmacokinetics and residues of clenbuterol in veal calves. *J. Anim. Sci.* 69:4538–4544.
14. Philippe, A. G., S. Marie-Claude, and H. S. Richard. 1999. Quantitative analysis of clenbuterol in meat products using liquid chromatography–electrospray ionisation tandem mass spectrometry. *J. Chromatogr. B* 736:209–219.
15. Podesta, A., A. Luppi, L. Benatti, and C. Villani. 1998. A source of false-negative results in clenbuterol analysis in tissues of veal calves. *J. Pharmacol. Toxicol. Methods* 40:27–32.
16. Posyniak, A., J. Zmudzki, and J. Niedzielska. 2003. Screening procedures for clenbuterol residue determination in bovine urine and liver matrices using enzyme-linked immunosorbent assay and liquid chromatography. *Anal. Chim. Acta* 483:61–67.
17. Pulce, C., D. Lamaison, G. Keck, C. Bostvironnois, J. Nicolas, and J. Descotes. 1991. Collective human food poisoning by clenbuterol residues in veal liver. *Vet. Hum. Toxicol.* 33:480–481.
18. Ramos, F., A. Cristino, P. Carrola, T. Eloy, J. M. Silva, M. C. Castilho, and M. I. N. Silveira. 2003. Clenbuterol food poisoning diagnosis by gas chromatography–mass spectrometric serum analysis. *Anal. Chim. Acta* 483:207–213.
19. Salleras, L., A. Dominguez, E. Mata, J. L. Taberner, I. Moro, and P. Salva. 1995. Epidemiologic study of an outbreak of clenbuterol poisoning in Catalonia, Spain. *Public Health Rep.* 110:338–342.
20. Sawaya, W. N., K. Lone, A. Husain, B. Dashti, and T. Saeed. 2000. Screening for β-agonists in sheep urine and eyes by an enzyme-linked immunosorbent assay in the state of Kuwait. *Food Control* 11:1–5.
21. Sporano, V., L. Grasso, M. Esposito, G. Oliviero, G. Brambilla, and A. Loizzo. 1998. Clenbuterol residues in non–liver containing meat as a cause of collective food poisoning. *Vet. Hum. Toxicol.* 40:141–143.
22. Whaites, L. X., and E. J. Murby. 1999. Determination of clenbuterol in bovine urine using gas chromatography–mass spectrometry following clean-up on an ion-exchange resin. *J. Chromatogr. B* 728: 67–73.
23. Wittmann, C., U. Bilitewski, T. Giersch, U. Kettling, and R. D. Schmid. 1996. Development and evaluation of a dipstick immunoassay format for the determination of atrazine residues on-site. *Analyst* 121:863–869.
24. Wittmann, C., and P. Y. Schreiter. 1999. Analysis of terbuthylazine in soil samples by two test strip immunoassay formats using reflectance and luminescence detection. *J. Agric. Food Chem.* 47:2733–2737.
25. Wong, R. 2002. The effect of adulterants on urine screen for drugs of abuse: detection by an on-site dipstick device. *Am. Clin. Lab.* 21: 37–39.
26. Yeoh, H. H., and F. Sun. 2001. Assessing cyanogen content in cassava-based food using the enzyme-dipstick method. *Food Chem. Toxicol.* 39:649–653.
27. Zhang, X. Z., Y. R. Gan, and F. N. Zhao. 2003. Determination of clenbuterol in pig liver by high-performance liquid chromatography with a coulometric electrode array system *Anal. Chim. Acta* 489:95–101.

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