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Determination of streptomycin residues in food by solid-phase extraction and liquid chromatography with post-column derivatization and fluorometric detection

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Abstract

A reliable and sensitive procedure is presented for the analysis of streptomycin (STP) in food of animal origin, like meat, milk and honey. The method is based on a separation by ion-pair liquid chromatography with β-naphthoquinone-4-sulfonate (NQS) postderivatization and fluorescence detection. The clean-up of the extract is done by solid-phase extraction, firstly with a cation-exchange cartridge and secondly with an octadecyl cartridge. The selectivity is very good and not many interfering peaks are observed for various food matrices. The streptomycin recovery of the total procedure is superior to 80%. The procedure is quantitatively characterized and repeatability, linearity, detection and quantification limits are very satisfactory. A special focus is given to STP residues in honeys and a survey on 64 commercial honeys is presented. For honey analysis, the HPLC method is compared with an immunoassay test (ELISA), and the possibility of using this test for screening with and without solid-phase extraction clean-up is also discussed.

Keywords: Streptomycin; Antibiotics

1. Introduction

Streptomycin (STP) is an aminoglycoside antibiotic used as a veterinary drug, especially in beekeeping, for the treatment of bacterial diseases, such as American or European foulbrood, in several countries (particularly Central and South America). Therefore, STP residues can be found in meat, liver, kidney, milk and above all in honey. If the STP concentrations found in food have no direct toxic effect, numerous allergic hypersensitivity cases were discovered during the last years [1], and STP can produce severe skin rashes in nurses. STP microbial resistance is a common finding and it has been shown that *E. coli*, *Salmonella* and *Shigella* may carry resistance [1]. For all these reasons, the control of streptomycin residues in food is necessary.

Consequently, a specific, sensitive and reliable analytical method is needed. Microbiological methods and Charm test are not sensitive or specific enough [2,3]. Immunological assays are very sensitive and can be used as screening tests, but results obtained with these assays must always be confirmed by a more selective method such as chromatography [2,3]. Streptomycin has no important chromophore

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groupments and is generally derivatized in order to obtain UV-absorbing or fluorescent compounds [4–9]. This derivatization step allows to reach a satisfactory sensitivity and selectivity for residues analysis in complex biological matrices, like honey, meat, liver, kidney or crude milk.

This paper presents an analytic procedure based on the works of Kocher [4] for the analysis of streptomycin in foods of animal origin (with a special focus on honey) by ion-pair liquid chromatography with β-naphthoquinone-4-sulfonate (NQS) post-column derivatization and fluorescence detection [8]. The clean-up of the extract is done by solid-phase extraction (SPE), firstly with a cation-exchange cartridge and secondly with an octadecyl cartridge. However, low recoveries have led us to optimize the sample handling steps and particularly the SPE clean-up.

The selectivity is very good and no problematic interfering peaks are observed for matrices, such as honey, crude milk, liver, kidney and meat. The procedure is quantitatively characterized and repeatability, linearity, detection and quantification limits are very satisfactory.

As mentioned above, we have focused on honey analysis, because streptomycin residues are a current and important problem. Results from the control of streptomycin residues in 64 commercial honeys from various countries are also presented.

As this procedure is relatively time consuming, a screening method could be useful. Therefore, for honey analysis, the HPLC method is compared with an immunoassay kit (ELISA), and the possibility of using this assay for screening with and without solid-phase extraction clean-up is also discussed.

2. Experimental

2.1. Apparatus

Sample handling is performed with Polytron PCU (Kinematica AG, Kriens, Switzerland) for homogenization and a Heraeus Megafuge 1.0 (Heraeus, Hanau, Germany) for centrifugation. The SPE clean-up steps are done with 200 mg sulfonic acid (Applied Separations, Allentown, USA) and 500-mg C_{18} (Malincrodt Baker, Germany) solid-phase extraction cartridges.

Liquid chromatography is performed with two Jasco (Tokyo, Japan) PU980 pumps, a fluorometric Jasco detector FL920, and a Jones Chromatography 7971 oven. The separation is done with a Hypersil BDS (100×4 mm, 3 μm) analytical column and an Hypersil ODS (4×4 mm, 5 μm) precolumn (Hewlett-Packard, Palo Alto, USA).

An SSI LC 300 pump (SSI, State College, USA) is used for post-derivatization with a PEEK connection after the analytical column. A tefzel reaction coil (1/16″×10 m length) is immersed in a thermostated bath at 55°C.

For ELISA assays a Ridascreen streptomycin kit (R-3101, purchased at Dispolab AG, Switzerland) is used with a spectrophotometer (450 nm) for the absorbance reading.

2.2. Reagents and chemicals

Hexane, terbutylmethylether (TBME) pro analysi, perchloric acid ($HClO_4$) 70%, glacial acetic acid, sodium hydroxide (NaOH) puriss, monohydrated sodium dihydrogenphosphate ($NaH_2SO_4 \cdot H_2O$), dihydrated disodium hydrogenphosphate ($Na_2HSO_4 \cdot 2H_2O$), sodium chloride (NaCl), sodium 1-heptanesulphonate (AHS) LiChropur, 1,2 naphthoquinon-4-sulphonic acid (NQS) sodium salt are all obtained from Merck (Darmstadt, Germany). Chromatography-grade acetonitrile (ACN) (Merck) is used for the HPLC mobile phase.

Streptomycin sesquisulfate (Sigma, St. Louis, MO, USA) is used as standard.

Certified reference honey containing 38 μg/kg of streptomycin (HKV1, Promochem GmbH, Molsheim, France) is used for accuracy determination.

PBS buffer for enzyme immunoassay is prepared by dissolving 0.55 g $NaH_2PO_4 \cdot H_2O$ and 2.85 g $Na_2HPO_4 \cdot 2H_2O$ and 8.7 g NaCl in 1 l water.

2.3. Method

2.3.1. HPLC method

The overall schematic procedure is presented in Fig. 1.

The extraction is performed on 5 g of sample, previously homogenized, for meat, liver and kidney

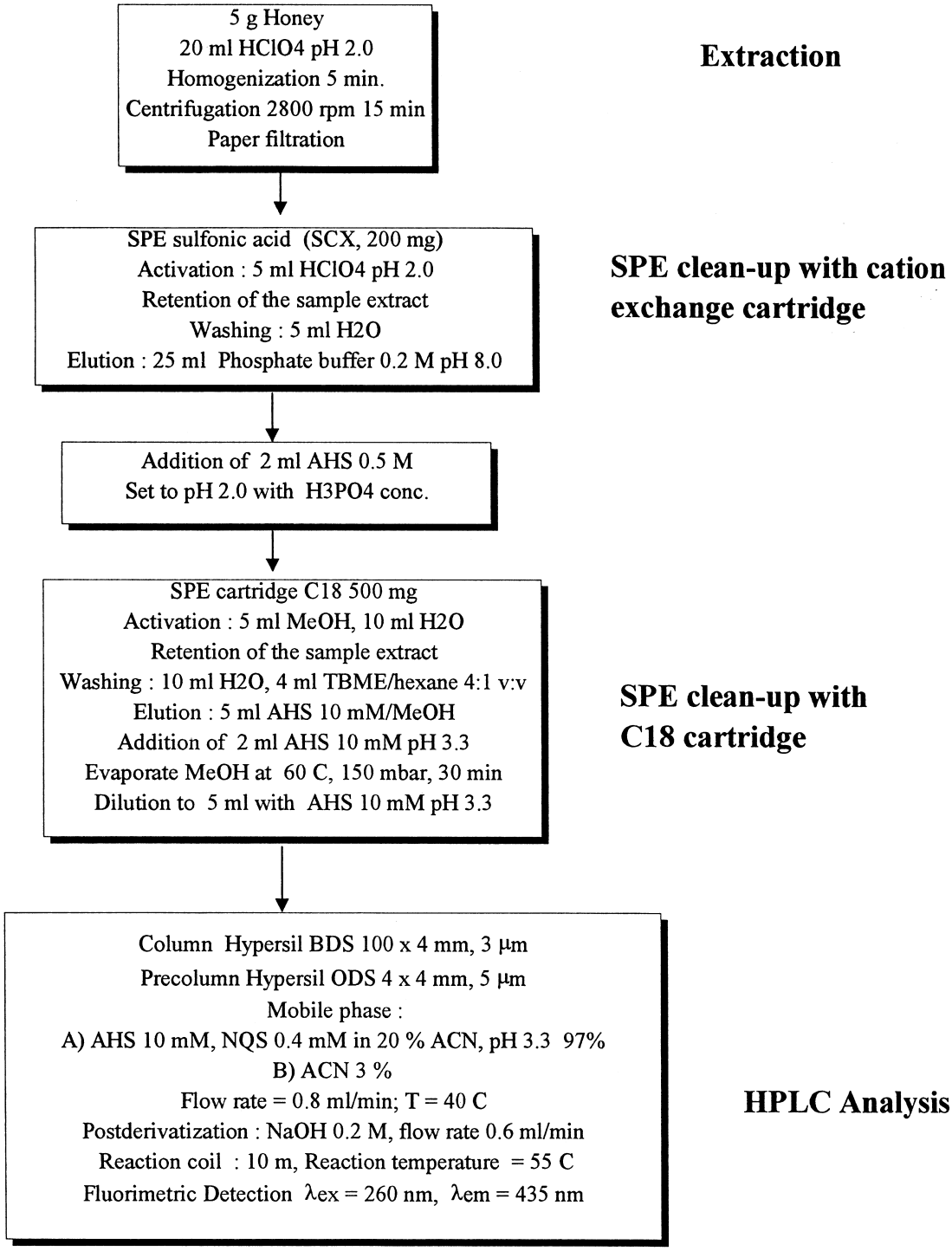

Fig. 1. Scheme of the analytical procedure.

and 20 ml perchloric acid at pH 2.0 (~0.01 *M*). The mixture is homogenized for 5 min with ultraturax, then centrifuged for 15 min at 2800 rpm. The liquid phase is collected and filtered on paper.

The first clean-up is made with cation-exchange cartridges, previously conditioned with 5 ml $HClO_4$, pH 2.0. The loading is done with the entire extract for honey and with an aliquot of 5 ml for crude milk and 4 ml for meat, liver and kidney samples.

After the sample extract loading, the phase is washed with 5 ml H_2O. Then, streptomycin is eluted with 25 ml 0.2 *M* phosphate buffer, pH 8.0. A 2-ml volume of AHS 0.5 *M* solution is added to the elutant and the pH is adjusted at 2.0 with concentrated phosphoric acid.

A C_{18} cartridge is conditioned with 5 ml MeOH and 10 ml 10 m*M* AHS, pH 3.3, and loaded with the sample extract. The C_{18} phase is washed with 10 ml H_2O, then with 4 ml solution of TBME/hexane (4:1, v:v). Elution is performed with 5 ml 10 m*M* methanolic AHS. As important losses in streptomycin content were observed when dry evaporation of MeOH was performed (maybe due to STP sublimation), 2 ml 10 m*M* AHS, pH 3.3, are added before methanol is evaporated (T=65°C, 100 mbar, during 30 min) The aqueous extract is finally collected and completed to a final volume of 5 ml with 10 m*M* AHS, pH 3.3.

2.3.2. Chromatographic conditions

The chromatographic separation is performed in isocratic mode with a mobile phase containing 97% 10 m*M* AHS, 0.4 m*M* NQS in 20% ACN and 3% ACN. In order to prevent NQS photodegradation, the mobile phase must be protected from light. The flow-rate is fixed at 0.8 ml/min and the column temperature regulated at 40°C. The injection volume is 100 μl. The post-column reaction is obtained with 0.2 *M* NaOH at 0.6 ml/min. The reaction temperature is 55°C with a 10-m reaction coil.

The fluorometric detection is done at 260 nm excitation and 435 nm emission wavelength. Fig. 2 presents the typical chromatogram obtained for a 100 μg/kg STP standard injection.

2.3.3. Enzyme immunoassay (ELISA) for honey analysis

If no clean-up is performed, 10 g of honey are mixed with 10 ml of water after homogenization. The solution is simply diluted 20 times with PBS buffer before the ELISA test.

In the other case, the clean-up is done by SPE as described for the HPLC method. The extract obtained after the C_{18} solid-phase extraction is only diluted five times with the PBS buffer.

The ELISA test is then done as described in the guidelines. Absorbance is read at 450 nm.

3. Results and discussion

3.1. Method performance

The method was validated following the Swiss manual for food analysis instructions [10], which are similar to the criteria of the European Union 93/256/EEC guidelines. The validation is done by the determination of the selectivity, repeatability, linearity, accuracy and the detection and quantification limits of the method.

The chromatograms, shown in Fig. 2, for various matrix extracts, clearly demonstrated that the method selectivity is very good for honey, meats and crude milk. Liver and kidney give more complex chromatograms, but STP concentrations corresponding to the Swiss legal allowance (1.0 mg/kg for kidney and 0.5 mg/kg for liver) are clearly observed and quantified. For comparison purposes, the STP MRLs (maximum residue levels) indicated in the European Union regulation 2377/90/EEC are higher: 0.3 mg/kg for muscle, 2.0 mg/kg for liver and 5.0 mg/kg for kidney.

Table 1 shows recoveries and repeatabilities observed for honey, crude milk, meat and liver analyses. Recoveries, about 80–90%, are very satisfactory and above all repeatabilities are very good, below 6% for the complete procedure.

The important step for good recoveries is the elution from the cation-exchange cartridge. Using conventional 500-mg cartridges or sometimes other sulfonic acid solid phases brands can give low recoveries.

The linearity of the method is determined by spiking honey with various STP quantities before the extraction. The method is linear for a streptomycin concentration range between 10 and 400 μg/kg with

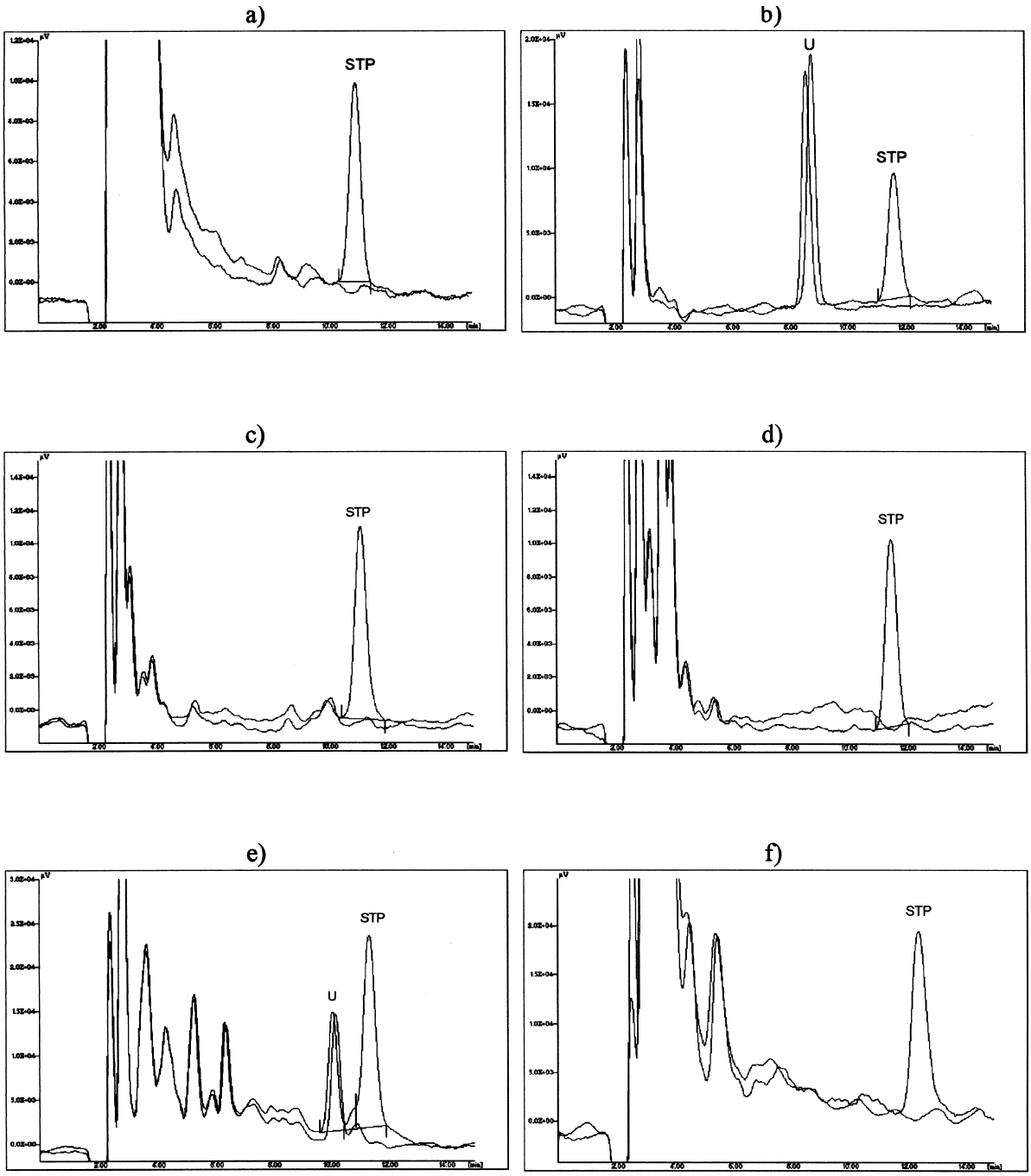

Fig. 2. Chromatograms of analyses of various food matrices with and without STP spiking: (a) honey (spiked with 100 μg/kg); (b) crude milk (spiked with 200 μg/kg); (c) beef meat (spiked with 500 μg/kg); (d) chicken meat (spiked with 500 μg/kg); (e) pig kidney (spiked with 1000 μg/kg); (f) pig liver (spiked with 500 μg/kg). STP, streptomycin; U, unknown.

Table 1
Streptomycin recoveries and repeatabilities

	Recovery (%)	Repeatability (%)
Milk (n=4)	88	5.6
Honey (n=7)	91	3.6
Meat (n=4)	81	4.8
Liver (n=4)	81	2.9

a regression coefficient of 0.99992. In the 10–400-μg/kg range, the analytical recoveries were 86–94%, with R.S.D. values of 0.4–3.5% (n=2 or 3).

The method accuracy was also determined with a certified reference honey sample containing 38 μg/kg streptomycin. The analysis was done in triplicate and by two different people.

The results show the good accuracy of this procedure (104% of the theoretical value) with a good repeatability (6%).

Table 2 gives the detection and quantification limits (determined with a signal-to-noise ratio of 3 for detection limit and 10 for quantification limit) in the different matrices, with the corresponding Swiss legal allowance values. In all cases, the sensitivity of the method is quite conform with the law requirements.

3.2. Streptomycin residues in honeys

As specified before, the method was particularly applied to commercial honeys. Actually, the STP Swiss allowance value is 100 μg/kg, whereas no MRL for STP in honey exists in the European Union regulation. These honeys (n=64) had various origins (Switzerland, France, Italy, Germany, Greece, South America, Mexico, USA, Canada, and some other countries), but many were in fact a mixture of honeys coming from different countries. The results obtained show that 29 samples (42%) contained STP residues. The measured STP concentrations included were between 10 and 160 μg/kg. Many honeys revealed STP concentrations inferior to the Swiss allowance value, except two samples with STP content of 138 and 163 μg/kg. It must be noted that South or Central America apiarists, or European importers, should make important efforts in order to reduce these STP residues before 2000, because the Swiss allowance value will decrease to 10 μg/kg.

Table 2
Limits of detection (LOD), limits of quantification (LOQ) and Swiss allowance values (AV) for streptomycin residues in food matrices

	LOD (mg/kg)	LOQ (mg/kg)	AV (mg/kg)
Honey	0.005	0.01	0.1
Milk	0.03	0.05	0.2
Meat	0.03	0.05	0.5
Liver	0.1	0.2	0.5
Kidney	0.1	0.2	1.0

It is equally interesting to consider the origin of honeys containing STP residues. Not only South or Central America honeys (seven samples), but also products purchased by co-operatives resulting from mixtures of various and indefinite origins (17 samples), with only a part coming from America, were almost all STP positive. Sometimes they even had higher STP residues than honeys coming exclusively from South or Central America. Finally, some European honeys (five samples) probably did not indicate the true origin, but only the conditioning place of the final product and also contained STP residues.

3.3. Comparison with ELISA assays

Chromatographic results obtained from 46 honey samples are compared with ELISA assays. Qualitative and quantitative bearings are studied in order to determine first, if ELISA can be used as a screening test for STP in honey, and second if quantitative results give a good correlation with the HPLC method.

In fact, a screening test is really advantageous if it can be used rapidly on a large sample number. This is the reason why, even though the manufacturer advocates a previous SPE clean-up of the honey extract, we have also studied the possibility of using ELISA with a simple buffer dilution of the honey. The qualitative bearing concerns, above all, the possibility of using ELISA as a screening test, and therefore we have determined if this test leads to false-positive and/or false-negative results.

Table 3 presents ELISA and HPLC results obtained for honey analyses with and without clean-up of the extract, and shows that a preliminary clean-up is absolutely necessary, because too many false positives (24%) are observed. In these conditions, ELISA is not reliable enough to be used as a

Table 3
Qualitative comparison for streptomycin residue analyses in honey by HPLC with ELISA with and without SPE clean-up

	ELISA without clean-up		ELISA with clean-up	
	Negative	Positive	Negative	Positive
HPLC-negative	6	11	15	2
HPLC-positive	0	29	0	29

screening test. On the other hand, no false-negatives are recorded.

After a SPE clean-up of the honey extract, ELISA results are quite in accordance with those observed by HPLC and almost no false-positives (4%) are recorded. However, as each positive ELISA result must anyway be confirmed by HPLC, the usefulness of this test is open to discussion. As a matter of fact, if we consider, on the one hand, the high cost of ELISA kits added to the cost of SPE cartridges, and on the other hand the long time needed for sample handling, this method seems finally really suitable only if a very large number of samples must be analyzed.

The quantitative bearing is studied only with results obtained on purified honey extracts.

Comparison between the HPLC and ELISA results indicated a relatively good correlation between both techniques. The straight line obtained had an intercept of 2.06 ± 2.24 and a slope equal to 1.07 ± 0.037 with a 0.988 coefficient of correlation. A Student *t*-test with a confidence interval corresponding to 95% showed that the intercept was not different to zero, and that the slope was not significantly different to 1.0.

The certified honey (containing 38 μg/kg of STP) ELISA analysis gives equally a very good result: 37 ± 4 μg/kg. However, the ELISA repeatability (C.V. ~11%, $n=6$) is less good than HPLC (C.V. ~6%), due to the logarithmic calibration, because weak absorbance variations lead to important disparities on the corresponding concentrations.

4. Conclusions

The developed HPLC method is selective and sensitive enough to permit STP residue analysis in various food matrices, like meats, liver, kidney, milk and honey. The repeatability, linearity range, detection and quantification limits are quite appropriate for STP analyses in food matrices in regards to the European and Swiss regulation requirements.

A special focus was done on STP residues in honey. A survey concerning 64 commercial honeys has shown that numerous samples contained STP residues (~42%), but generally with concentrations lower than the actual Swiss allowance value (0.1 mg/kg). However, it would be very interesting to continue these analyses in the near future, because the allowance concentration value will progressively decrease to 0.01 mg/kg in January 2000.

For honey analysis, the HPLC method was compared with an ELISA test. ELISA results obtained with purified honey extracts showed a good correlation with HPLC. However, in this case, using ELISA as a screening test seems not really advantageous, considering the high financial cost and the time needed.

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