Talanta 178 (2018) 934–942

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Flow-batch analysis of clenbuterol based on analyte extraction on molecularly imprinted polymers coupled to an in-system chromogenic reaction. Application to human urine and milk substitute samples

Natalia González[a], Marcos Grünhut[a], Ivana Šrámková[b], Adriana G. Lista[a], Burkhard Horstkotte[b], Petr Solich[b], Hana Sklenářová[b], Carolina C. Acebal[a,*]

[a] INQUISUR, Departamento de Química, Universidad Nacional del Sur (UNS)-CONICET, Av. Alem 1253, 8000 Bahía Blanca, Argentina
[b] Charles University, Faculty of Pharmacy in Hradec Králové, The Department of Analytical Chemistry, Heyrovského 1203, 500 05 Hradec Králové, Czech Republic

ARTICLE INFO

Keywords:
Clenbuterol
Flow-batch analysis
Molecularly imprinted polymer
Solid phase extraction
Diazotization-coupling reaction

ABSTRACT

A fully automated spectrophotometric method based on flow-batch analysis has been developed for the determination of clenbuterol including an on-line solid phase extraction using a molecularly imprinted polymer (MIP) as the sorbent.

The molecularly imprinted solid phase extraction (MISPE) procedure allowed analyte extraction from complex matrices at low concentration levels and with high selectivity towards the analyte. The MISPE procedure was performed using a commercial MIP cartridge that was introduced into a guard column holder and integrated in the analyzer system. Optimized parameters included the volume of the sample, the type and volume of the conditioning and washing solutions, and the type and volume of the eluent.

Quantification of clenbuterol was carried out by spectrophotometry after in-system post-elution analyte derivatization based on azo-coupling using N- (1-Naphthyl) ethylenediamine as the coupling agent to yield a red-colored compound with maximum absorbance at 500 nm. Both the chromogenic reaction and spectrophotometric detection were performed in a lab-made flow-batch mixing chamber that replaced the cuvette holder of the spectrophotometer.

The calibration curve was linear in the 0.075–0.500 mg L^{-1} range with a correlation coefficient of 0.998. The precision of the proposed method was evaluated in terms of the relative standard deviation obtaining 1.1% and 3.0% for intra-day precision and inter-day precision, respectively. The detection limit was 0.021 mg L^{-1} and the sample throughput for the entire process was 3.4 h^{-1}.

The proposed method was applied for the determination of CLB in human urine and milk substitute samples obtaining recoveries values within a range of 94.0–100.0%.

1. Introduction

Clenbuterol (CLB) is a sympathomimetic drug selectively acting on β-2 receptors used as a decongestant and bronchodilator. Therapeutically, CLB is applied in the treatment of breathing disorders, such as bronchial asthma or obstructive pulmonary disease, or even in respiratory allergies and infections with bronchospasm. Due to its relaxing effect on non-striated muscles, CLB can also be used as a tocolytic agent for farm animals [1]. In addition, exceeding therapeutic doses, CLB has the ability to increase muscle mass and reduce body fat. For this reason, it has been used illegally as an anabolic agent by athletes and bodybuilders and as a growth-promoter in livestock to increase meat production [2].

Various methods have been proposed for the determination of CLB mainly in biological samples using separation techniques. Particularly, LC-MS techniques have been extensively used, in general as part of multi-residue methods [3,4]. Recently, UHPLC-MS/MS was employed to determine CLB in human urine and pork tissue samples [5,6]. Faster analysis of aqueous and urine samples was achieved using ion mobility spectrometry [7]. Additionally, methods based on capillary electrophoresis [8,9] with amperometric and mass detections have also been published. Moreover, specific determination of CLB was also reported using non-separation methods such as time-resolved fluoroimmunoassay [10] and enzyme-linked immunosorbent assay [11], which required highly costly reagents.

Despite the increasing complexity of the instrumentation used to

* Corresponding author.
E-mail address: cacebal@uns.edu.ar (C.C. Acebal).

http://dx.doi.org/10.1016/j.talanta.2017.10.040
Received 23 August 2017; Received in revised form 16 October 2017; Accepted 20 October 2017
Available online 23 October 2017
0039-9140/

develop analytical methods, approaches based on spectrophotometry remain on the table due to their universality, simplicity, low cost, and availability in all analytical laboratories. In addition, these methods are very simple to automate and detection can be performed in flow cells or flow-batch chambers located directly in the spectrophotometer, or using modular UV–Vis detectors with optical fibers. Due to the limited selectivity, spectrophotometric determination of CLB has been most often performed following HPLC with UV or DAD detectors [12–14].

When analyzing complex matrices, sample preparation is essential to enable reliable detection at low concentration levels. From the wide variety of available preparation techniques, solid phase extraction (SPE) is one of the most often chosen because it is relatively simple to perform, it requires low amounts of organic solvents, and it yields high preconcentration efficiency and excellent sample throughput. Moreover, SPE is easily automated reducing the analysis time and avoiding the operator's exposure to potential hazards. Due to the non-specificity of conventional sorbents for a single target compound, it is likely to co-elute interfering compounds with similar physicochemical characteristics as the analyte of interest. Because of that, the use of molecularly imprinted polymer (MIP) as the sorbent, so-called molecularly imprinted solid-phase extraction (MISPE), presents important advantages over conventional sorbents since they contribute with high selectivity due to their specific recognition over the target analyte, and the possibility to perform a customized sample treatment before determination. Moreover, the use of MIPs allows for improving the sensitivity of the analytical methods by diminishing potential matrix effects that occur in the final determination, and thus reducing the background noise compared with conventional sorbents [15,16]. Extraction and preconcentration of CLB by using MIP, whether commercial or lab-synthetized, has been described for the analysis of mainly urine and animal tissues samples [14,17]. In addition, some sensors based on the use of MIPs that allow for the simple selective recognition of CLB have been reported [18,19]

The introduction of flow injection analysis (FIA) has been the initial point for the development of new techniques and novel methods in which analytical procedures are performed partially or fully automatically [20]. Within the wide range of available techniques used to automate analytical procedures, flow-batch analysis (FBA) combines the main advantages of the batch approach (e.g. low effects of sample viscosity, temperature, reaction kinetics, etc.) with the great potential of multi-commutated flow analysis [21]. The main component of the FBA system is the mixing chamber, into which all solutions are propelled so that a wide variety of processes can be carried out, including sample preparation, reagent addition, mixing, and detection [22]. Thus, FBA offers an excellent alternative for the automation of analytical procedures owing to its flexibility and versatility, which gives it multitasking features. The flow of the solutions into and out of the chamber is controlled by solenoid valves so that only the required amount of each one is used, which makes FBA an environmentally-friendly methodology that minimizes reagent consumption and waste production.

FBA has mostly been used to develop several analytical applications based on either selective chromogenic reactions [23,24] or in combination with chemometric approaches [25], potentially including SPE analyte preconcentration [26].

In this work, a fully automated FBA system for the spectrophotometric determination of CLB in human urine and milk substitute samples was developed based on selective analyte preconcentration by MISPE and coupled to in-chamber post-elution derivatization. For this, a diazotization reaction [27,28] using clenbuterol as the diazotizable compound was performed to produce an intensely colored azo dye. The aromatic amine group of clenbuterol reacts with nitrite ions in acid medium to give rise to the formation of a diazonium cation. This salt reacts with N- (1-Naphthyl) ethylenediamine (NED, coupling agent) forming an azo compound with maximum absorbance at 500 nm. To the best of our knowledge, this is the first time that CLB has been determined by this reaction.

While the color reaction guarantees measurement sensitivity, the selectivity towards the analyte is given by the MISPE procedure. The proposed system thus allows performing extraction, preconcentration, colorimetric reaction, and detection in a completely automated way.

2. Experimental

2.1. Reagents

Analytical grade reagents and ultra-pure water ($> 18\ M\Omega\ cm^{-1}$) were used throughout the experiments. Methanol, MeOH (99.8%, HPLC grade, Merck, Germany) and acetonitrile, ACN (≥ 99%, Merck, Germany) were used as the solvents for MISPE.

A 200 mg L^{-1} clenbuterol hydrochloride (Sigma-Aldrich, Germany) stock solution was prepared in MeOH and stored in a dark bottle at 4 °C. An intermediate standard solution of 30 mg L^{-1} was prepared daily by diluting the appropriate volume of the stock solution in MeOH. Working standard solutions ranging from 0.075 to 0.500 mg L^{-1} were prepared by appropriately diluting the intermediate standard with a 0.025 mol L^{-1} of ammonium acetate solution with pH 6.7.

A trichloroacetic acid, 1.0% (w/v), TCA (Merck, Germany) solution was prepared by weighting 0.50 g and subsequent dilution with MeOH up to a volume of 50.0 mL. Likewise, 1.0% (w/v) trifluoroacetic acid TFA (Merck, Germany) was prepared.

A solution of 0.025 mol L^{-1} ammonium acetate, NH_4Ac (Anedra, Argentina) with pH 6.7 was prepared by dissolving 0.0964 g of the salt into 50.0 mL of water.

For the diazotization reaction, a 0.025 mol L^{-1} sodium nitrite solution and a 6×10^{-3} mol L^{-1} N-(1-Naphthyl)ethylenediamine, NED (Carlo Erba, Argentina) solution were prepared in 50% (v/v) MeOH. In addition, a 4.6 mol L^{-1} hydrochloric acid, HCl (37%, Merck, Germany) solution was prepared in water. Adequate dilutions of each of these solutions were prepared daily.

A 0.10 mol L^{-1} sodium dodecylsulfate, SDS (Anedra, Argentina) solution was prepared. For this, 0.765 g of SDS was dissolved and diluted with water up to 25.0 mL.

SupelMIP® SPE-Clenbuterol cartridges (bed wt. 25 mg, volume 10 mL) were purchased from Sigma Aldrich (53201-U SUPELCO) [31] and used for the MISPE procedure. The column comprised a commercial cartridge (syringe type) that was cut at the level of the frits and introduced into a Chromolith® 5-4.6 guard cartridge holder (Merck).

2.2. Apparatus and software

Spectrophotometric measurements were taken out using an Agilent 8453 spectrophotometer (integration time: 0.5 s; interval: 1 nm). The cuvette holder was replaced by a fit-for-purpose mixing and detection chamber (MDC) designed in our laboratory. A scheme and dimensions of the MDC are depicted in Fig. 1. It consisted of a PTFE block with a dead volume of 1.2 mL and four lateral inlets (for three reagents and eluate) and one outlet for emptying at the bottom. Moreover, the MDC presented two oppositely positioned optical glass windows (10 mm diameter) in such a way that the MDC allowed for photometric measurements. In order to obtain a rapid and efficient mixture of the solutions, a stirring system was designed and assembled on top of the MDC. This was done by coupling a PTFE blade vertically to a microprocessor ventilator (Intel®, DC 12 V, 28 G-forces).

All the solutions were pumped into the flow-batch analysis (FBA) system by a Gilson® Minipuls 3 peristaltic pump (PP1). An Ismatec mini-S 420 peristaltic pump (Cole-Parmer GmbH, Wertheim, Germany) was used as the auxiliary pump (PP2) to empty the MDC. Three-way solenoid valves (Type, NResearch®) were used to dispense the appropriate volumes and handle the solutions into the system.

MHLL Tygon® tubes with an i.d. of 1.14 mm, suitable for organic solvents, were used for all pumping channels. All tubing to solution

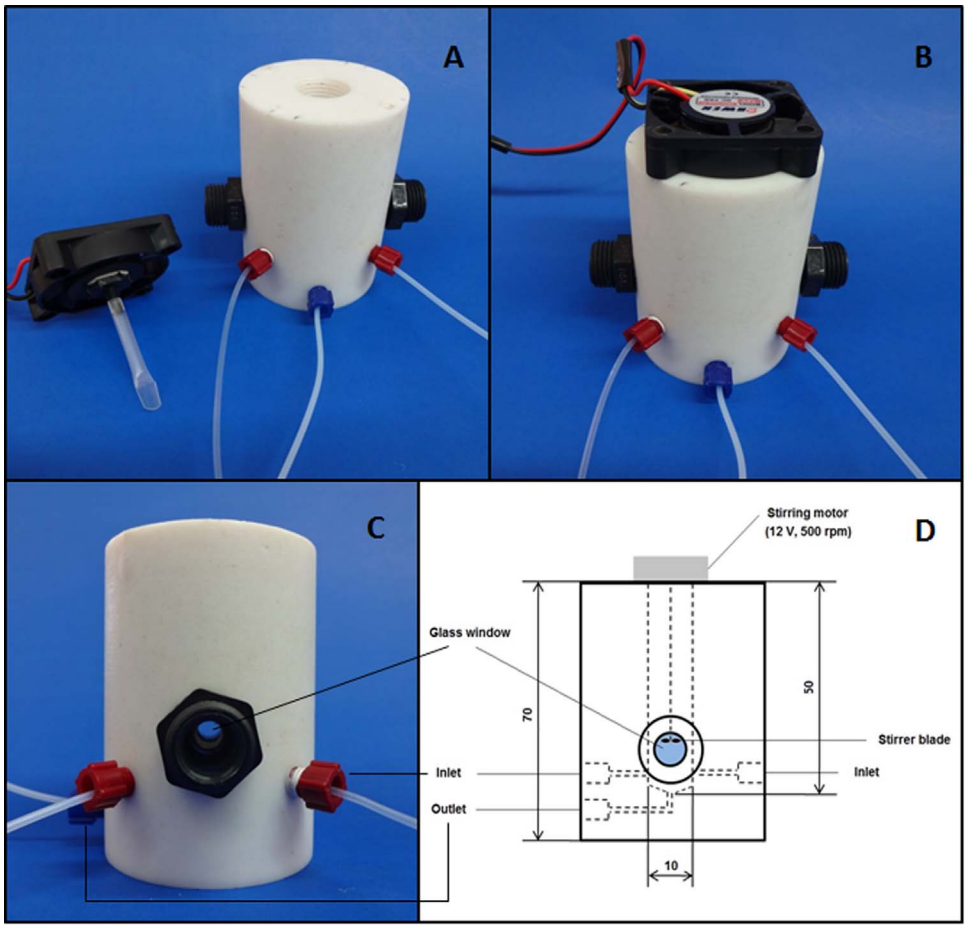

Fig. 1. Pictures, schematic, and dimensions of the MDC designed and built in our laboratory. A, B: General view of the MDC and the stirrer system; C: Lateral view of the MDC; D: Schematic diagram of the lateral view. Dimensions are expressed in mm.

reservoirs and ¼" UNF 28/" thread-connectors in the mixing and detection chamber were made of PTFE (0.5 mm i.d.).

A flow rate of 0.75 mL min^{-1} was selected for the MISPE procedure as well as for all the solutions flowing toward the mixing chamber. A flow rate of 1.35 mL min^{-1} was selected for emptying the chamber.

The program used to control the FBA system was developed in the LabVIEW® 5.1 development system (National Instruments) and controlled the peristaltic pump, the solenoid valves, and the stirrer device through a 7-output electronic actuator board (EA) that was connected to a Pentium® 4 microcomputer.

2.3. Operation of the automatic analysis

Fig. 2 shows the diagram of the proposed MISPE-FBA system. For better understanding, the automatic procedure and system are explained in two parts: the preconcentration by MISPE and posterior spectrophotometric detection. Table 1 shows the switching time intervals for the complete procedure.

2.3.1. Preconcentration procedure

The preconcentration part of the system consisted of four solenoid valves (V1–V4) and four channels (C1–C4). C1 corresponded to water or a buffer with pH 6.7, C2 to the sample, C3 to a solution of ACN 70% and C4 to MeOH or TCA 1% in MeOH.

Each day, the MIP cartridge was conditioned using 1.0 mL of MeOH and washed with 1.0 mL of water passed for this purpose through channels C1 and C4. Since these steps were performed at the beginning of the working day, the solutions in C1 (water by the buffer solution) and C4 (MeOH by TCA 1% in MeOH) were changed manually when the corresponding valves were OFF. Next, an equilibrating step was performed by passing 1.0 mL of NH_4Ac buffer solution (pH 6.7) through the cartridge via C1. After this, 5.0 mL of sample were pumped through the MIP cartridge through C2 by switching V1 to the ON position. Before elution, a washing step was performed using 2.0 mL of 70% (v/v) ACN solution (C3) by switching V3 to the ON position. Finally, the elution step was performed with 0.50 mL of 1% (v/v) TCA in MeOH. For this step, V2 and V3 were switched ON and the eluent solution was pumped in C4 through the MIP cartridge. For all pre-elution steps, V4 was switched OFF so that all solutions were pumped to waste after passing through the MIP. During the elution step, V4 was switched ON to direct the eluate containing the CLB toward the MDC.

2.3.2. Spectrophotometric detection procedure

The MDC allowed for mixing the eluate with the reagents for both the colorimetric reaction and spectrophotometric detection.

The part of the automated system used for the post-MISPE reaction required three channels (C5 to C7) for solutions of 1.5×10^{-3} mol L^{-1} $NaNO_2$ (C5), 4.6 mol L^{-1} HCl (C6) and 6.5×10^{-4} mol L^{-1} NED (C7). The flow rates were set at 0.75 mL min^{-1}. The direction of the flow in the channels was controlled by three solenoid valves (V5–V7). When the corresponding valve was switched OFF, the solution was recycled to the respective container; when the valve was ON, the respective solution was pumped into the MDC. Immediately after the entry of the eluate - containing the analyte (0.50 mL) - into the MDC, V5 was switched ON and 0.125 mL of $NaNO_2$ reagent was propelled into the MDC. Simultaneously, the stirring system was activated. Then, V6 and V7 were sequentially switched ON and 0.300 mL of HCl and 0.250 mL of NED were propelled into the MDC, respectively. The stirring device was activated and the mixture was then stirred for 15 s. Then, the reaction product was detected at 500 nm. After this, the MDC was emptied by switching ON the auxiliary peristaltic pump (PP2).

Finally, the MDC was cleaned by activating the stirring system and

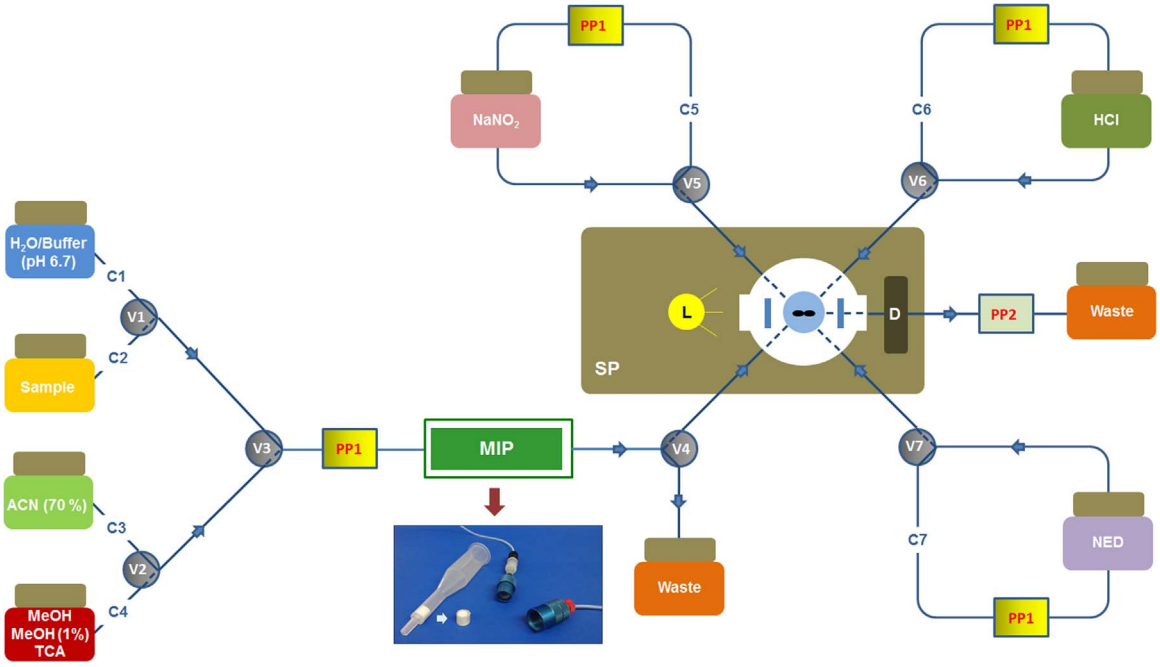

Fig. 2. MISPE-FBA system for extraction, preconcentration, and spectrophotometric determination of CLB. V1-V7: solenoid valves (the solid line represents the OFF position and the dotted line represents the ON position); C1-C7: flow channels; PP1: peristaltic pump; PP2: auxiliary peristaltic pump; MIP: Molecularly imprinted polymer column; SP: spectrophotometer; L: light source; D: detector.

switching ON V4 to fill the MDC with an additional 2.0 mL of 1% (v/v) TCA in MeOH and then emptying it by activating PP2.

2.4. Sample preparation

Human urine samples were obtained from three healthy donors of both genders and processed immediately. They were centrifuged (Rolco 2036, Argentina) for 20 min at 44 G-forces to remove sediments and then filtered through a 0.22 µm syringe filter. Then, 12.5 mL of the treated sample were diluted with the NH_4Ac buffer with pH 6.7 to a final volume of 25.0 mL and introduced into the automatic analysis system.

Commercial samples of cow milk substitute were acquired from veterinary stores in Bahía Blanca, Argentina. They were prepared according to the manufacturer's instructions by dissolving 8.00 g of the product in 60.0 mL of water at 50–55 °C and then adding cold water to a final volume of 100.0 mL. Afterwards, 20.0 g of ammonium sulfate (Anedra, Argentina) were added to the sample to achieve precipitation of milk proteins. Next, the sample was centrifuged for 20 min at 448 G-forces and the supernatant was filtered through an 8-µm pore width Whatman™ filter paper. The sample was then diluted by a factor of 2 with an NH_4Ac buffer, pH 6.7, and introduced into the automatic analysis system.

To study analyte recovery, the untreated samples were spiked with CLB at two concentration levels within the following calibration range: 0.200 and 0.350 mg L^{-1} and the same preparation protocol was

Table 1
Switching time intervals for the MISPE-FBA system.

	Switching time intervals (s)									
	V_1	V_2	V_3	V_4	V_5	V_6	V_7	PP	PP_A	MS
Preconcentration steps										
Conditioning										
Methanol	OFF	80	80	OFF	OFF	OFF	OFF	80	OFF	OFF
Water	OFF	OFF	OFF	OFF	OFF	OFF	OFF	80	OFF	OFF
Equilibration										
Buffer (pH 6.7)	OFF	OFF	OFF	OFF	OFF	OFF	OFF	80	OFF	OFF
Sample loading										
Urine or milk	400	OFF	OFF	OFF	OFF	OFF	OFF	400	OFF	OFF
Washing										
ACN (70%)	OFF	OFF	160	OFF	OFF	OFF	OFF	160	OFF	OFF
Elution										
TCA 1% in MeOH	ON	27	27	27	OFF	OFF	OFF	27	OFF	OFF
Determination steps										
Color reaction										
$NaNO_2$ (1.5×10^{-3} mol L^{-1})	OFF	OFF	OFF	OFF	8	OFF	OFF	8	OFF	8
HCl (4.6 mol L^{-1})	OFF	OFF	OFF	OFF	OFF	20	OFF	20	OFF	20
NED ($6.5 \times$ 10–4 mol L^{-1})	OFF	OFF	OFF	OFF	OFF	OFF	15	15	OFF	15
Detection	OFF	OFF	OFF	OFF	OFF	OFF	OFF	OFF	OFF	OFF
Emptying of chamber	OFF	OFF	OFF	OFF	OFF	OFF	OFF	OFF	35	OFF
Cleaning of chamber										
Water	OFF	OFF	OFF	120	OFF	OFF	OFF	120	OFF	120

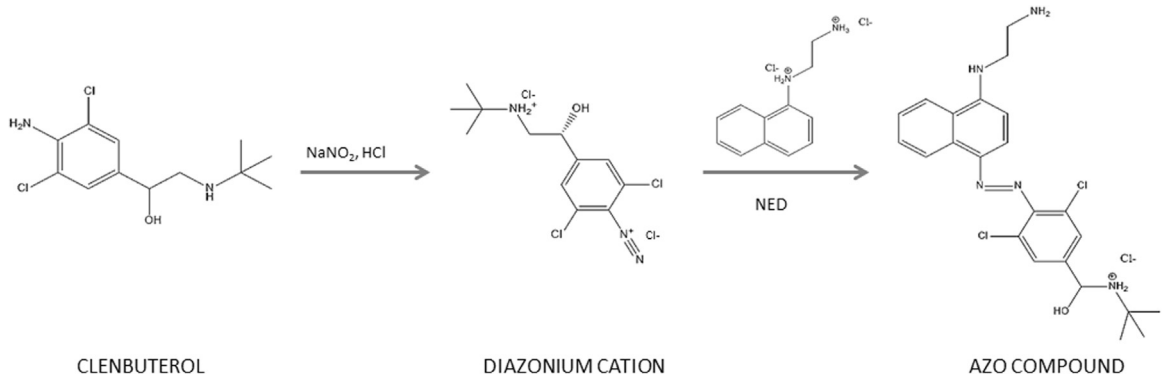

Fig. 3. Diagram of the diazotization-coupling reaction of CLB.

followed. Recovery values were then calculated according to the AOAC definition [29]. All analyses of the samples were performed in triplicate.

3. Results and discussion

3.1. Optimization of the colorimetric reaction

The determination of CLB was performed using a diazotation-coupling method employing NED as a coupling agent. The reaction is shown in Fig. 3, and the formed azo compound can be measured spectrophotometrically at 500 nm.

The optimization of the experimental conditions for the colorimetric reaction was performed, and the optimal values aiming for the highest absorbance signal and the best repeatability of the measurements were chosen.

3.1.1. Concentration and volumes of the reagents

Both the concentration and the volume of the individual reagent aiming for the highest signal and minimal volume to perform the detection with the MDC to avoid eluate dilution were optimized.

Fig. 4 shows the obtained results for the study of each reagent. It was found that blank signals were constantly low in all studies. With respect to the concentration of the HCl solution, an interval of 2.3–6.0 mol L^{-1} was tested obtaining an optimal value of 4.6 mol L^{-1} at an optimum volume of 300 µL. Concerning the $NaNO_2$ solution, concentrations were tested between 5.0 and 2.25 mmol L^{-1}, and 1.5 mmol L^{-1} was selected for a volume of 125 µL.

Different concentrations of NED solution were prepared in the range of 0.16–1.6 mmol L^{-1}. The highest signal with the minimum volume was obtained with 250 µL of a 0.65 mmol L^{-1} solution.

The optimized volumes and concentrations were used for further experiments.

3.1.2. Order of addition of the reagents

Four possible orders of adding CLB, HCl and $NaNO_2$ solutions were tested while maintaining the addition of the coupling NED agent at the end of the sequence: (1) CLB-HCl-$NaNO_2$, (2) $NaNO_2$-CLB-HCl, (3) $NaNO_2$ + HCl-CLB, and (4) CLB-$NaNO_2$-HCl. No significant variation of the spectrophotometric signal was observed between these sequences. Thus, to follow the same order of addition for all experiments, the reaction was performed using the first sequence.

3.1.3. Effect of the reaction temperature

As it is well known, diazotization reactions are both time- and temperature-dependent. Thus, both parameters were studied.

The influence of the reaction temperature was tested between 20 °C (room temperature) and 40 °C. The reaction was performed offline and the absorbance was measured after heating the reaction mixture in a water bath from 0 to 7 min. It was found that temperatures higher than

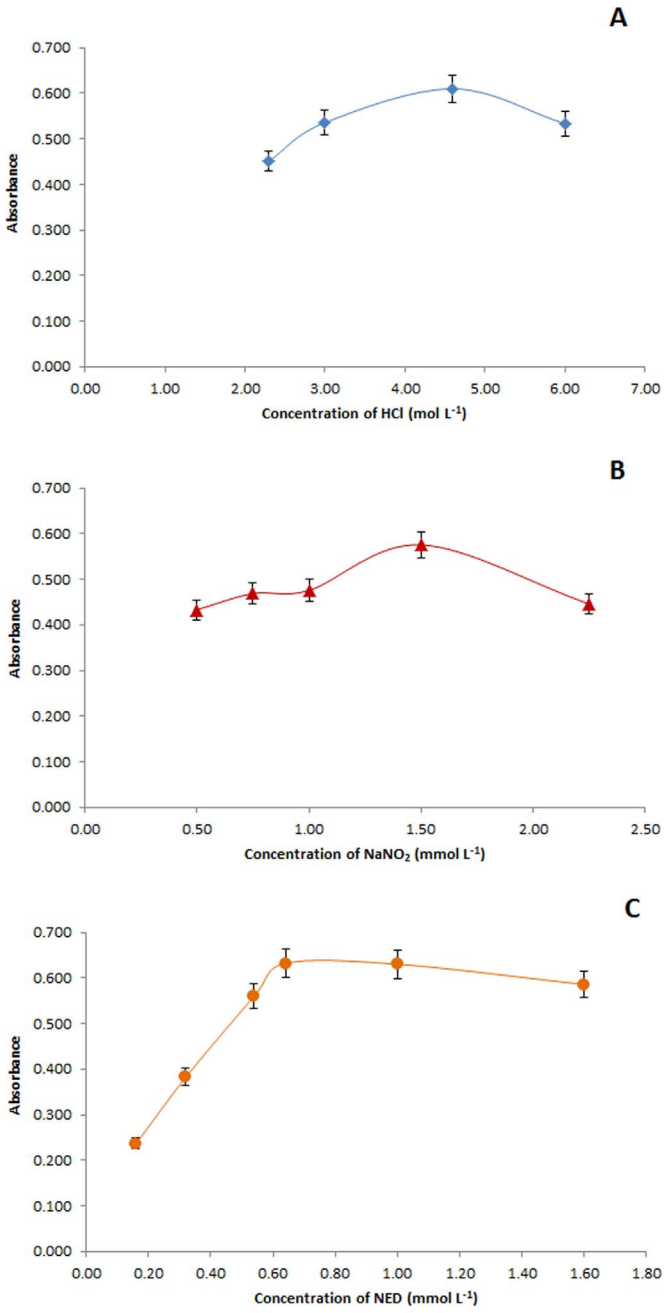

Fig. 4. Optimization of the concentration of the reagents of the diazotization-coupling reaction of CLB. A: HCl concentration for a volume of 300 µL; B: $NaNO_2$ concentration for a volume of 125 µL; C: NED concentration for a volume of 250 µL.

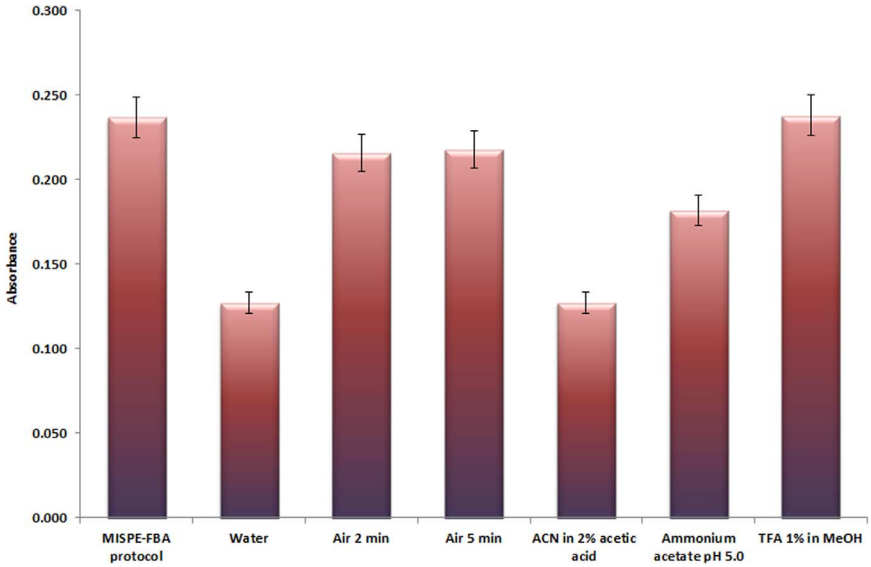

Fig. 5. Evaluation of the different steps recommended by the MIPs' manufacturer using the proposed MISPE-FBA method.

37 °C inhibited color development, which is in agreement with former observations [25]. A slight but not significant increase of the signal at temperatures between 20 °C and 35 °C was observed as a function of time. Thus, the laboratory temperature (20 °C) was used for further experiments.

Color development aiming for high sensitivity was studied as a function of time. Monitoring the reaction over 15 min at room temperature showed an incremental signal during the first 4 min. After that period, the signal remained constant, which demonstrates reaction steady-state and stability for at least 15 min. Therefore, a 4-min interval was selected to perform further measurements.

3.1.4. Addition of the SDS solution

As has been suggested in literature [30], the addition of a micellar solution can improve color development by increasing the coupling reaction rate. Thus, 100 µL of 8.2×10^{-3} mol L^{-1} SDS solution were added after the addition of the CLB solution. The concentration of the reagents was adjusted to maintain the relation between concentrations and the final volume in the reaction (1.275 mL). No significant increase in reaction rate was observed with the addition of SDS.

3.2. Optimization of the MISPE process

Clenbuterol is found in low concentrations in both human urine and milk substitute samples. In addition, the analyzed samples present a complex matrix. For this reason, an extraction and preconcentration procedure was performed.

3.2.1. MISPE- protocol

According to the procedure recommended by the MIP manufacturer [29], once the sample is loaded, salts and water-soluble matrix components are washed out with 1.0 mL of water. Then, 2 min of vacuum is necessary to semi-dry the polymer and afterwards, 1.0 mL 2% acetic acid in ACN is passed through the cartridge to remove acidic compounds bonded by both ionic and hydrophobic interactions. Subsequently, 1.0 mL of a 0.5 mol L^{-1} NH_4Ac pH 5.0 buffer solution is used to remove strong basic and hydrogen-bonded interferences. Finally, 1.0 mL of 70% ACN in water is used to break both hydrophobic and hydrogen bonds.

For the present work, the original MISPE protocol was modified to reduce the number of steps and reagents taking into account that the performed colorimetric reaction increased the determination selectivity. To estimate the method performance for eliminating interference, MISPE optimization was performed directly using human urine and milk substitute samples, processed as described in Section 2.4 and spiked with 0.350 mg L^{-1} of CLB.

Each of the recommended steps was evaluated and the results obtained were compared with those obtained when performing the complete procedure.

Compared with the original MISPE protocol, modifications were included into the post-loading washing steps and prior to elution.

A comparison of the obtained results for the optimization of the washing steps is shown in Fig. 5. As can be observed, a higher absorbance signal was obtained for the MISPE-FBA protocol when the washing was performed with 2.0 mL of 70% ACN in water. When the column was washed with 1.0 mL of water and 1.0 mL of ACN in 2% acetic acid, the signal significantly decreased. The vacuum step, which was proposed by the manufacturer, was replaced by an air stream that flowed through the column for 2 and 5 min, and no considerable difference in the signals was observed. Finally, the washing step in which NH_4Ac was passed through the column also showed a slight decrease in the signal.

The volume of 70% ACN in water was optimized between 1.0 and 4.0 mL, obtaining satisfactory results (recovery percentages over 90%) at 2.0 mL. For the elution of CLB, 1% (v/v) TFA and 1% (v/v) TCA solutions prepared in MeOH were tested as eluents and compared. Similar results were obtained for both organic acids, and 1% TCA solution was selected due to the lower toxicity.

Therefore, the complete optimized protocol for the MISPE-FBA method consisted of a conditioning step using 1.0 mL of MeOH and washing with 1.0 mL of water. Next, an equilibrating step was performed by pumping an NH_4Ac buffer solution (pH 6.7) through the column. Next, 5.0 mL of sample were loaded into the MIP, and afterwards, a washing step was performed using 2.0 mL of ACN (70%). Finally, the elution step was performed with 0.50 mL of a 1% (v/v) TCA in MeOH.

3.2.2. Sample volume and flow-rate

With the aim to improve the enrichment factor, the effect of the loading volume of the sample was studied taking into account the analytical response and the time of analysis. Volumes from 1.50 to 20.0 mL of a 0.500 mg L^{-1} CLB standard solution were loaded onto the MIP column following the optimized procedure. It was observed that the absorbance increased linearly with the volume of the sample, but the time of analysis was also significantly higher. As a compromise between sample throughput and the method's sensitivity, a 5.0 mL volume was chosen. Hence, an enrichment factor of 10 was yielded, but higher values are possible if required.

Table 2
Analytical parameters obtained by the proposed MISPE-FBA method.

Analytical parameters	Obtained value
Linear range (mg L^{-1})	0.075–0.500
Slope (mg L)$^{-1}$	0.637 ± 0.029
Intercept	0.025 ± 0.008
R^2	0.998
LOD (mg L^{-1})	0.021
Intra-day Precision (RSD %)[a]	1.1
Inter-day Precision (RSD %)[b]	3.0
Sample throughput (h^{-1})	3.4

[a] n = 8.
[b] Measure by triplicate over 3 days; LOD calculated as 3 *s*/A, where, *s*: standard deviation. A: slope of the calibration curve.

The influence of the loading flow rate on the signal was studied and 0.75 mL min^{-1} was chosen as the maximum flow rate that can be used due to overpressure problems in the preconcentration part of the system.

3.3. Analytical performance

The analytical performance was evaluated in terms of the linearity, limit of detection (LOD), sample throughput, and intra-day and inter-day precision. The results are summarized in Table 2.

The optimized FBA method was used to construct calibration curves for the CLB determination over a range of 0.075–0.500 mg L^{-1} (five points with three replicates). The regression equation was A = (0.637 ± 0.029) [CLB mg L^{-1}] + (0.025 ± 0.008) with a correlation coefficient of 0.998. The LOD value was 0.021 mg L^{-1} and was calculated from the calibration function.

The intra-day precision was evaluated by RSD (%) values obtained with a 0.35 mol L^{-1} CLB solution and 8-fold repetition, while for inter-day precision, the same concentration was measured in triplicate on 3 consecutive days. The obtained RSD values of 1.1% and 3.0% proved high repeatability and inter-day precision of the automatic method for both the extraction and photometric determination steps.

The sample throughput of the optimized method was 3.4 h^{-1} including MISPE, reaction, detection and MDC washing, and this was comparable to LC-MS determination [7].

3.4. Analysis of real samples

Clenbuterol is a prohibited substance and there is no tolerated level of consumption. The substance is taken to promote muscle growth and to increase the performance of athletes [32]. CLB is excreted by urine so that doping and illegal consumption is tested in human urine samples.

In addition, CLB is also administered to livestock to increase meat production, but this practice is also prohibited [33]. One of the most common and effective ways to illegally administer CLB is through animal feed. Due to the fact that calves are mainly fed by milk up to 6 months of age, high doses of CLB are added to milk substitutes during reconstitution immediately before feeding the animals daily [34]. The proposed method was therefore applied to the determination of CLB in human urine as well as milk substitute samples.

Thus, three human urine samples (A, B and C), collected randomly from healthy volunteers in sterile plastic flasks and two samples of commercial milk substitutes (D and E) were analyzed. The optimal conditions for the preparation of the samples are discussed in the Supplementary information, and the results are shown in Fig. S1.

No residues of CLB were found in the selected samples above the method's LOD. Thus, the samples were spiked at two concentration levels of CLB within the following calibration range: 0.200 and 0.350 mg L^{-1}, treated as described, and introduced into the MISPE-FBA system for their analysis. Fig. 6 shows the spectra of the 0.350 mg L^{-1} standard solution, non-spiked samples and spiked samples at the 0.350 mg L^{-1} level. The results of the recovery study ranged from 94.0% to 99.2% and 94.0 to 100.0% for the human urine and milk substitute samples, respectively, and are summarized in Table 3.

The obtained results were satisfactory, demonstrating the capacity and the versatility of the method to be applicable to complex samples

Table 3
Determination of CLB in urine and milk substitute samples.

Sample	Added (mg L^{-1})	Found (mg L^{-1})	R (%)
A	0.200	0.188 ± 0.005	94.0
	0.350	0.341 ± 0.014	97.5
B	0.200	0.196 ± 0.005	97.8
	0.350	0.331 ± 0.008	94.5
C	0.200	0.189 ± 0.001	94.6
	0.350	0.347 ± 0.006	99.2
D	0.200	0.200 ± 0.010	100.0
	0.350	0.329 ± 0.008	94.0
E[a]	0.200	0.195 ± 0.003	97.5
	0.350	0.339 ± 0.022	96.8

The samples were analyzed in triplicate. A,B,C: urine samples; D,E: milk substitute samples.
[a] Sample E contains oxytetracycline and neomycin sulfate in unknown quantities.

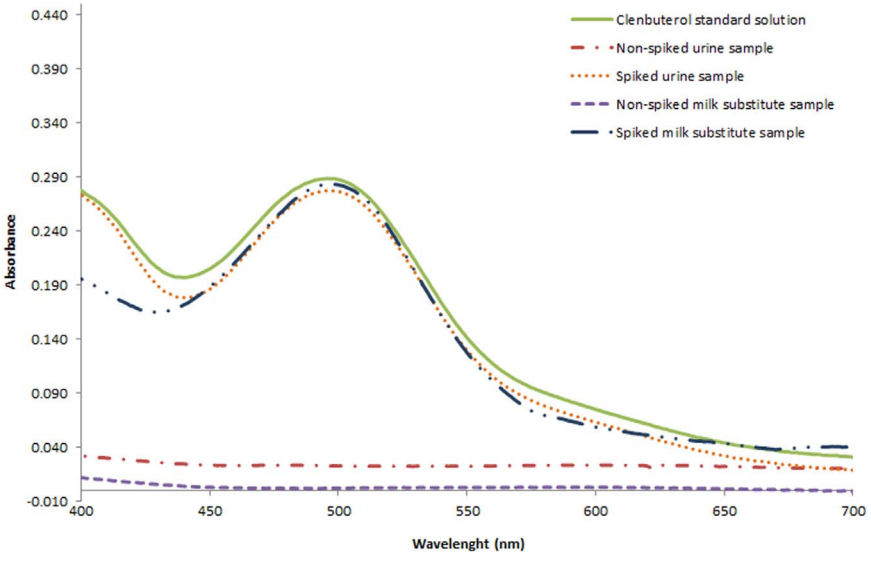

Fig. 6. Spectra of the 0.350 mg L^{-1} standard solution (——), non-spiked urine (— · ·) and milk substitute (— — —) samples, and spiked urine (·····) and milk substitute (——) samples at 0.350 mg L^{-1} level.

even in the presence of other drugs, such as antibiotics, in its formulation (Sample E).

According to literature, the common doses of CLB to be administered to calves to promote growth ranges from 10 and 20 $\mu g\,kg^{-1}$ per day [35,36]. Thus, taking into account that calves' weight increases from 30 to 160 kg from birth to weaning and that they consume around 4.0–6.0 L (at the higher weight) of milk substitute per day, the concentration of CLB to be added to the prepared samples ranges from 0.075 to 0.270 $mg\,L^{-1}$. Therefore, the proposed method is adequate to determine CLB in milk substitute samples since the target concentrations lay within the calibration range.

In the case of urine samples, the detection limit of the proposed MISPE-FBA method is higher than the methods found in literature whose values varied between 5×10^{-4} and $2 \times 10^{-3}\,mg\,L^{-1}$ [6,7,14]. In spite of that, the loading volume of the sample can be increased or alternatively, a long-path detection cell could be used to increase the method's sensitivity. Moreover, it must be pointed out that most methods propose a second preconcentration step by evaporating the eluate and reconstituting it into a small volume. Since it was proven that the colorimetric reaction is suitable for determining CLB in urine, these strategies could be applied to improve the sensitivity of the presented method.

3.5. Conclusions

The proposed MISPE-FBA method was successfully applied for the determination of CLB in human urine and milk substitute samples, resulting in a decreased consumption of reagents, very good reproducibility of the sampling, handling, and measurements, and an acceptable sample throughput for the complete procedure.

The proposed configuration allowed for performing SPE based on a molecular imprinted polymer, with subsequent fully-automated online colorimetric derivatization reaction and quantitation providing satisfactory measurement repeatability and reproducibility. The proposed method proved high versatility as it was possible to determine the analyte in two very different matrices and with acceptable accuracy and analyte recovery in both cases.

The use of the MIPs as sorbents resulted in a selective extraction of the analyte with few steps required for sample preparation (deproteination, centrifugation, and filtration). In addition, the number of procedural steps in the original MISPE procedure was decreased by employing azo-coupling with a gain in both selectivity and sensitivity. This also led to a decreased volume of solvents used and waste generated. Moreover, a preconcentration factor of 10 was achieved.

In addition, a novel spectrophotometric method for the determination of CLB was proposed as a low-cost, easy-to-implement, and rapid alternative to the methods that can be found in literature, where determination is mainly performed by chromatographic techniques.

Acknowledgments

N. González, M. Grünhut, A. Lista and C. Acebal also would like to express their gratitude to Universidad Nacional del Sur (24/Q086) and Consejo Nacional de Investigaciones Científicas y Técnicas, CONICET (11220120100625KE5).

I. Šrámková, P. Solich and H. Sklenářová appreciate financial support of the project STARSS reg. No.: CZ.02.1.01/0.0/0.0/15_003/0000465 co-funded by ERDF.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2017.10.040.

References

[1] M. Parr, G. Opfermann, W. Schäänzer, Analytical methods for the detection of clenbuterol, Bioanalysis 1 (2009) 437–450.
[2] G. Garssen, G. Geesink, A. Hoving-Bolink, J. Verplanke, Effects of Dietary clenbuterol and salbutamol on meat quality in veal calves, Meat Sci. 40 (1995) 337–350.
[3] L. Wang, Z. Zeng, Z. Wang, L. He, Correction function for biased results due to matrix effects in residue analysis of beta-agonists in porcine tissues and urine with LC-MS/MS, RSC Adv. 6 (2016) 26967–26976.
[4] J. Domínguez-Romero, J. García-Reyesa, R. Martínez-Romero, E. Martínez-Lara, M. Del Moral-Leal, A. Molina-Díaz, Detection of main urinary metabolites of β2-agonists clenbuterol, salbutamol and terbutaline by liquid chromatography high resolution mass spectrometry, J. Chromatogr. B 923–924 (2013) 128–135.
[5] L. Chu, S. Zheng, B. Qu, S. Geng, X. Kang, Detection of β-agonists in pork tissue with novel electrospun nanofibers-based solid-phase extraction followed ultra-high performance liquid chromatography/tandem mass spectrometry, Food Chem. 227 (2017) 315–321.
[6] R. Nicoli, M. Petrou, F. Badoud, J. Dvorak, M. Saugy, N. Baume, Quantification of clenbuterol at trace level in human urine by ultra-high pressure liquid chromatography–tandem mass spectrometry, J. Chromatogr. A 1292 (2013) 142–150.
[7] O. Peñuela-Pinto, S. Armenta, F. Esteve-Turrillas, M. de la Guardia, Selective determination of clenbuterol residues in urine by molecular imprinted polymer—ion mobility spectrometry, Microchem. J. 134 (2017) 62–67.
[8] Y. He, X. Li, P. Tong, M. Lu, L. Zhang, G. Chen, An online field-amplification sample stacking method for the determination of β2-agonists in human urine by CE-ESI/MS, Talanta 104 (2013) 97–102.
[9] L. Li, H. Du, H. Yu, L. Xu, T. You, Application of ionic liquid as additive in determination of three β-agonists by capillary electrophoresis with amperometric detection, Electrophoresis 34 (2013) 277–283.
[10] M. Lia, Y. Zhang, Y. Xue, X. Hong, Y. Cui, Z. Liu, D. Du, Simultaneous determination of β2-agonists clenbuterol and salbutamol in water and swine feed samples by dual-labeled time-resolved fluoroimmunoassay, Food Control 73 (2017) 1039–1044.
[11] Y. Tang, J. Gao, X. Liu, X. Gao, T. Ma, X. Lu, J. Li, Ultrasensitive detection of clenbuterol by a covalent imprinted polymer as a biomimetic antibody, Food Chem. 228 (2017) 62–69.
[12] Y. Tang, J. Lan, X. Gao, X. Liu, D. Zhang, L. Wei, Z. Gao, J. Li, Determination of clenbuterol in pork and potable water samples by molecularly imprinted polymer through the use of covalent imprinting method, Food Chem. 190 (2016) 952–959.
[13] G. Brambilla, M. Fiori, B. Rizzo, V. Crescenzi, G. Masci, Use of molecularly imprinted polymers in the solid-phase extraction of clenbuterol from animal feeds and biological matrices, J. Chromatogr. B 759 (2001) 27–32.
[14] A. Blomgren, C. Berggren, A. Holmberg, F. Larsson, B. Sellergren, K. Ensing, Extraction of clenbuterol from calf urine using a molecularly imprinted polymer followed by quantitation by high-performance liquid chromatography with UV detection, J. Chromatogr. A 975 (2002) 157–164.
[15] A. Speltini, A. Scalabrini, F. Maraschi, M. Sturini, A. Profumo, Newest applications of molecularly imprinted polymers for extraction of contaminants from environmental and food matrices: a review, Anal. Chim. Acta 974 (2017) 1–26.
[16] O. Shimelis, A. Trinh, C. Aurand, Molecularly Imprinted Polymer SPE Increases Sensitivity for the Extraction and Analysis of Clenbuterol from Urine, Reporter US Volume 25(2), 2007. 〈https://www.sigmaaldrich.com/technical-documents/articles/reporter-us/molecularly-imprinted0.html〉. (Accessed 17 August 2017).
[17] W. Dua, C. Lei, S. Zhang, G. Bai, H. Zhou, M. Sun, Q. Fu, C. Chang, Determination of clenbuterol from pork samples using surface molecularly imprinted polymers as the selective sorbents for microextraction in packed syringe, J. Pharm. Biomed. 91 (2014) 160–168.
[18] Y. Tang, Z. Gao, S. Wang, X. Gao, J. Gao, Y. Ma, X. Liu, J. Li, Upconversion particles coated with molecularly imprinted polymers as fluorescence probe for detection of clenbuterol, Biosens. Bioelectron. 71 (2015) 44–50.
[19] F. Feng, J. Zheng, P. Qin, T. Han, D. Zhao, A novel quartz crystal microbalance sensor array based on molecular imprinted polymers for simultaneous detection of clenbuterol and its metabolites, Talanta 167 (2017) 94–102.
[20] M. Trojanowicz, K. Kołacińska, Recent advances in flow injection analysis, Analyst 141 (2016) 2085–2139.
[21] P.H. Goncalves Dias Diniz, L. Farias de Almeida, D.P. Harding, M.C. Ugulino de Araújo, Flow-batch analysis, Trends Anal. Chem. 35 (2012) 39–49.
[22] E. Zagatto, J. Carneiro, S. Vicente, P. Fortes, J. Santos, J.L.F.C. Lima, Mixing chambers in flow analysis: a review, J. Anal. Chem. 64 (2009) 524–532.
[23] M. Lima, S. Andrade, M. Silva Neta, I. Barreto, L. Almeida, M. Ugulino de Araújo, A micro-flow-batch analyzer using webcam for spectrophotometric determination of ortho-phosphate and aluminium(III) in tap water, J. Braz. Chem. Soc. 25 (2014) 898–906.
[24] E.A. Lima, M.B. Lima, S. Andrade, I. Barreto, M. Ugulino de Araújo, R.C. Lima, L. Almeida, A micro-flow-batch analyzer using an in-line cadmium sponge microcolumn for the photometric determination of nitrate and nitrite in dairy samples, Food Anal. Methods 7 (2014) 1925–1931.
[25] D. Fernandes, A. Gomes, M.M. de Fontes, G.B. da Costa, V. de Almeida, M. Ugulino de Araújo, R. Galvão, G. Véras, UV-Vis spectrometric detection of biodiesel/diesel blend adulterations with soybean oil, J. Braz. Chem. Soc. 25 (2014) 169–175.
[26] C. Acebal, M. Grünhut, I. Šrámková, P. Chocholouš, A. Lista, H. Sklenářová, P. Solich, B. Fernández Band, Application of a fully integrated photodegradation-detection flow-batch analysis system with an on-line preconcentration step for the determination of metsulfuron methyl in water samples, Talanta 129 (2014) 233–240.
[27] G. Norwitz, P. Kellher, Spectrophotometric determination of aniline by the

diazotization-coupling method with 8-amino-1-hydroxynaphthalene-3,6-disulfonic acid as the coupling agent, Anal. Chem. 53 (1981) 56–60.
[28] G. Norwitz, P. Kellher, Spectrophotometric determination of aniline by the diazotization-coupling method with N-(I-Naphthy1)ethylenediamine as the coupling agent, Anal. Chem. 53 (1981) 1238–1240.
[29] AOAC Peer-Verified Methods Program, Manual on Policies and Procedures, Arlington, VA, USA, 1998.
[30] G. Ramis Ramos, J. Esteve Romero, M. García Alvarez-Coque, Colorimetric determination of arylamines and sulphonamides by diazotization and coupling in a micellar solution, Anal. Chim. Acta 223 (1989) 327–337.
[31] Sigma Aldrich technical note, Extraction of the Beta-Agonist Clenbuterol from Urine Using Clenbuterol SupelMIP 〈https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Supelco/The_Reporter/eu_reporter_25_spe.pdf〉. (Accessed 17 August 2017).
[32] Prohibited list, World anti-doping agency 〈https://www.wada-ama.org/en/questions-answers/2013-prohibited-list#item-366〉. (Accessed 17 August 2017).
[33] SENASA, Resolución 60/01 Sagpa (PUBLICADA EN EL B.O. No29.577 DEL 30/01/2001).
[34] I. Dürsch, A. Malucelli, H. Meyer, Screening of clenbuterol, salbutamol and terbutaline in animal feeds, Food Agr. Immunol. 6 (1994) 141–145.
[35] P. Williams, l. Pagliani, G. Innes, K. Pennie, C. Harris, P. Garthwait, Effects of a P-agonist (clenbuterol) on growth, carcass composition, protein and energy metabolism of veal calves, Br. J. Nutr. 57 (1987) 417–428.
[36] V. Schindler, I. Feola, Comparación del peso desde el nacimiento hasta el destete de terneros de biotipos Braford y cuarterones en la región del NEA, Argentina, Zootec. Trop. 30 (2012) 327–333.