Journal of Pharmaceutical and Biomedical Analysis 52 (2010) 694–700

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Solid surface spectroscopic methodology for ultra-trace urinary nickel monitoring in smokers and non-smokers' subjects

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ARTICLE INFO

Article history:
Received 10 November 2009
Received in revised form 4 February 2010
Accepted 9 February 2010
Available online 16 February 2010

Keywords:
Urinary nickel
Chemical enrichment
Solid surface fluorescence
Active and passive smokers
Stability urine sample

ABSTRACT

Nickel chemical enrichment on nylon membranes previously treated with eosin (eo) is proposed for subsequent quantification by spectrofluorimetry (λ_{em} = 547 nm, λ_{exc} = 515 nm). Operational variables which have influence on quantitative metal retention have been studied. At optimal experimental conditions, quantitative recovery was reached (superior to 99%), with a detection limit of 0.13 ng L^{-1} and quantification limit of 0.44 ng L^{-1}. The calibration sensitivity was of 6×10^{13} ng L^{-1} for the new methodology with a linear range of 0.44–410 ng L^{-1} Ni(II). The tolerance levels, respect to cations and anions as potential interferents, were studied, with good results. The methodology was validated by standard addition method and satisfactorily applied to urinary nickel determination of 50 subjects including smokers, second hand smokers and non-smokers' samples without previous treatment. Stability of biological samples was daily studied for a period of 1 month. Within-day precision was better than 0.02 CV. The reproducibility (between-day precision) was also evaluated over 3 days by performing six determinations each day with a CV of 0.052. The different groups were evaluated using one-way analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparison test with satisfactory results.

1. Introduction

Smoking habit represents the main cause of human disease and death, feasible to prevent. In Argentine, tabaquism has the highest level of South America causing 40,000 death/year. Passive smokers or second hand smoke (SHS) exposure is an associated problem to tobacco addiction; fortunately some cities have regulations to protect non-smokers in working and public places; however, 55% of young people are exposed in their own homes [1].

There are numerous harmful substances in tobacco; among these substances, several toxic metals are found and may be acquired through active and passive smoking. Nickel is an essential metal to human life; nevertheless, nickel compounds are human carcinogens by inhalation. On the basis of epidemiological studies, EPA (Agency of Environmental Protection) classified nickel dust as a class A carcinogen [2].

Smokers are exposed to nickel compounds in quantities varying between 2 and 6.2 μg/cigarette. Approximately 10–20% of metal is expelled in the cigarette smoke and can be inhaled as nickel carbonyl, a very toxic substance. Inhalation is the most significant route of nickel exposure with regard to lung effects. Retention in the respiratory tract is more important than uptake into the general circulation. Inhibition of 5′-nucleotidase activity and enhanced lipid peroxidation in pulmonary alveolar macrophage have been demonstrated in the respiratory tract after injecting nickel chloride in rats [3].

Blood is the ideal matrix for most chemicals due to its contact with the whole organism and its equilibria with organs and tissues where chemicals are stored. However, it has the important disadvantage of needing an invasive sampling. Plasma and urine nickel concentrations have shown to be useful biomarkers of nickel inhalation exposure [4]. The development of new methodologies and modern analytical techniques has allowed the use of other matrices that are less or non-invasives [5].

Because of the low concentration level of analite in biological fluids, the introduction of a preconcentration step previous to instrumental detection results indispensable. Solid phase extraction (SPE) is a rapid, simple, economical preconcentration step, environmentally friendly than the traditional liquid–liquid extraction. SPE followed by electrothermal atomic absorption spectrometry (ETAAS), inductively coupled plasma atomic emission (ICP-AES) or ICP-mass spectrometry are suitable for analysis of nickel traces [6–11].

The need of more selective systems for separation of metal ions has produced important developments of chemical separation techniques. Nowadays, the researchers are interested in improving the selective preconcentration of the sorbents used

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doi:10.1016/j.jpba.2010.02.013

in SPE. This objective is particularly important when analysing complex matrices such as biological samples. Several solid materials as filter papers, silica gel, exchange resins, aluminium oxides, poly(vinylalcohol), C18 membranes, cyclodextrines, between others have been successfully employed as supports for SPE. Recently, nylon has proved to be an adequate support for luminescent detection of organic compounds [12–16]. Experimental results shown that this support possesses good selectivity, low background signal and can be used without previous treatment.

In this work, the separation/chemisorption of nickel on nylon membranes previously treated with eo, is proposed for the subsequent quantification by molecular fluorescence. The study was carried out analysing the different factors which have influence on the chemisorption step and fluorescent signal of the nickel–eo association and was applied to nickel determination in urine samples of smokers, second hand smokers and non-smokers. Urinary nickel determinations were carried out in the same sample for a period of 1 month, in order to apport specific information related to nickel stability in the biological samples.

2. Experimental

2.1. Reagents

Urine samples were tested using Urine Strip-Wiener Lab. (Rosario, Argentina).

1×10^{-9} mol L^{-1} Ni (II) stock solutions were prepared by dilution of 1000 μg mL^{-1} standard solution plasma-pure (Leeman Labs, Inc.).

1×10^{-2} mol L^{-1} Tris (Mallinckrodt Chemical Works, NY, Los Angeles, St. Louis, USA) solution was prepared. This solution was adjusted to the desired pH, with aqueous $HClO_4$ (Merck, Darmstadt, Germany) or NaOH (Mallinckrodt Chemical Works, NY, Los Angeles, St. Louis, USA).

1×10^{-7} mol L^{-1} eosin stock solution (H.E - Daniel Ltd., England) was weekly prepared by dissolution of the appropriate amount in ultrapure water.

All used reagent were of analytical grade.

2.2. Apparatus

Urinary sediments were typified using Eclipse E200 Microscopy (Nikon Instruments, Inc.).

Nylon membranes (Millipore, Sao Paulo, Brazil) 0.45 μm pore size and 47 mm diameter were used in chemisorption studies.

Fluorescence measurements were done using a Shimadzu RF-5301 PC spectrofluorometer equipped with a 150 W Xenon lamp and solid sample holder with GF-UV35 filter. Instrument excitation and emission slits were both adjusted to 3 nm.

A combined glass electrode and a pHmeter (Orion Expandable Ion Analyzer, Orion Research, Cambridge, MA, USA) Model EA 940 were used for pH adjustments.

A centrifuge was used in biological samples processing.

A Gilson Minipuls 3 peristaltic pump with PVC pumping tubes coupled to an in-line filter holder 47 mm (Millipore) was use for filtrating urine/standard solutions.

The microphotography was taken from a screening electron microscopy model LEO 450 VP (Carl Zeiss).

All used glass materials were previously washed with a 10% (v/v) HNO_3 water solution and then with ultrapure water.

2.3. Subjects of study

Studied subjects were interrogated using a written test, in order to obtain information related to smoking habit (frequency, time of addiction, etc.), age, sex, occupational situation, etc.

2.4. Biological sample collection

The first morning urine samples of 50 subjects were collected from occupationally unexposed subjects. Biological samples were collected in nickel-free polystyrene test tubes, between 8 and 10 h to reduce possible circadian contributions. Samples were centrifuged for 10 min at $1000 \times g$ and processed immediately after arriving to laboratory. None stabilizer is recommended to add because of the risk of incorporating analyte as impurity [17]. The obtained samples (10 mL approximately each sample) were centrifuged for 10 min; the sediments (2 mL approximately for each sample) were studied by optical microscopy. Supernants (5 mL approximately) were reserved for nickel quantification.

2.5. Dilution test

In order to establish the adequate dilution of each sample for applying determination methodology, dilution test was carried out as follow.

5 mL of each biological sample were taken and dilutions were carried out to obtain dilution factors of 1/2, 1/4, 1/8, 1/16 and 1/20.

2.6. Physical characterization and semi-quantitative determination of clinical parameters in biological samples

Biological samples were physically characterized, namely colour, odor and appearance, presence of sediment, blood and mucus, in order to stablish variables that could affect the obtained results.

Urine samples were tested using commercial reagent strips and sediments were observed using an optical microscopy.

2.7. Protection of human subjects

Written informed consent was obtained from all participants.

2.8. General procedure

Nylon membranes were impregnedated in batch by contact with 1×10^{-7} mol L^{-1} eo solution for 5 min. Membranes were dried at ambient temperature and reserved in dried ambient (20–25 °C) up to filtration step. Later, a dried membrane was put in filtration holder.

125–5000 μL of urine/standard solution (4.40×10^{-4}–0.41 μg L^{-1}), 100 μL of buffer Tris solution 1×10^{-2} mol L^{-1} (pH = 7.0), were placed in a 10 mL graduated centrifuge tube. The whole mixture was diluted to 10 mL with ultrapure water. Systems were filtrated across eo-impregned membranes, using peristaltic pump at 0.25 mL min^{-1} and dried at ambient temperature.

Nickel was determined on the membranes by fluorescent emission at λ_{em} = 547 nm and λ_{exc} = 515 nm, using a solid sample holder.

2.9. Interferences study

Different amounts of ions (1/1, 1/10, 1/100 and 1/1000 Ni(II)/interferent ratio) were added to the test solution containing 29 ng L^{-1} Ni(II) and general procedure was applied.

2.10. Accuracy study

5 mL of urine samples were spiked with increasing amounts of Ni(II) (29–587 ng L^{-1}). Nickel contents were determined by proposed methodology.

2.11. Precision study

The repeatability (within-day precision) of the method was tested for urine replicate samples ($n = 6$) spiked with 29 ng L^{-1} of nickel and metal contents were determined by proposed methodology.

2.12. Recovery procedure

5 mL of urine was spiked with increasing amounts of Ni(II) (29–587 ng L^{-1}) at five levels and treated following proposed methodology.

2.13. Stability test of biological samples

5 mL of biological samples were spiked with increasing amounts of Ni(II) (29–587 ng L^{-1}). Nickel contents were determined by proposed methodology at different times (1 day, 5 days, 1 week, 2 weeks, 1 month after sampling) using preservation in refrigerator at 277 K.

3. Results and discussion

3.1. Preliminary studies

Eosin yellowish [2′,4′,5′,7′-tetrabromo-3′,6′-di-hydroxyspiro [isobenzofuran-1(3H),9′-[9H]xanthen]-3-one] is a xanthene dye with strong absorption in the visible region. Previous researches have shown that the eo dye presents the feasibility of forming ternary complexes with metallic ions [18,19]. The fluorescent emission of eo–Ni(II) system was initially explored using SPE and determining the fluorescent signal of formed association on solid support.

Different potentially useful solid supports were put in contact in batch experiments with eo solution, in order to assure the effective retention of the dye. Metal retention was investigated passing nickel solution through the previously prepared membranes. In Table 1, the obtained results are shown. The retention levels for each assayed material were checked measuring solid fluorescence signals at $\lambda_{em} = 547$ nm, using $\lambda_{exc} = 515$ nm. The best results were obtained with nylon membrane.

Table 1
Study of retention of eo dye and nickel on different types of membranes.

Type of membrane	Observations	Relative fluorescence
Cellulose acetate		
(Whatman)	Eo retention: (+)	0.28
Pore size: 0.45 μm	Ni (II) retention: (−)	0.20
Immobilon (+)		
(Millipore)	Eo retention: (+)	0.27
Pore size: 0.45 μm	Ni (II) retention: (−)	0.20
Teflon		
(Millipore)	Eo retention: (−)	–
Pore size: 1 μm	Ni (II) retention: (−)	–
Mixed esters		
(Schleicher & Schuell)	Eo retention: (−)	–
Pore size: 0.45 μm	Ni (II) retention: (−)	–
Filter paper		
(S&S)	Eo retention: (+/−)	0.13
Black ribbon	Ni (II) retention: (−)	0.15
Filter paper		
(S&S)	Eo retention: (+/−)	0.17
Blue ribbon	Ni (II) retention: (−)	0.10
Nylon		
(Millipore)	Eo retention: (+)	0.30
Pore size: 0.45 μm	Ni (II) retention: (+)	1.00

Ni(II) concentration = 29 ng L^{-1}; eo concentration = 1×10^{-7} mol L^{-1}.

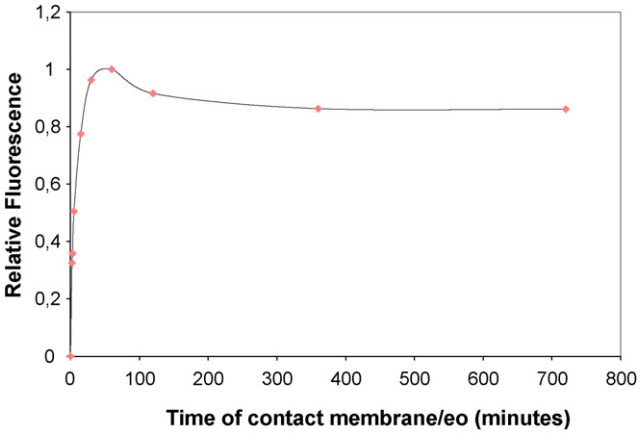

Fig. 1. Influence of contact time membrane/eo. Membrane of nylon; eo concentration 1×10^{-7} mol L^{-1}.

3.2. Effect of eosin concentration

In order to assure quantitative Ni(II) retention, assays were carried out varying the eo concentration between 1×10^{-9} and 1×10^{-3} mol L^{-1}. Concentration of 1×10^{-7} mol L^{-1} was selected as optimal, which is sufficiently high to warrant an eo excess with respect to expected Ni(II) contents in biological samples.

3.3. Membrane/eo contact time

Nylon membranes were put in batch contact with eo solution for different periods of time. Fig. 1 shows the obtained results. 5 min was chosen as optimal contact time, being the minimal time that enabling quantitative eo retention.

3.4. Influence of filtration flow rate

In order to optimize the number of processed samples/time unit, the filtration rate was varied between 0.05 and 0.25 mL min^{-1}, maintaining constant other experimental conditions. A filtration flow rate of 0.25 mL min^{-1} resulted the most appropriated for the quantitative eo retention (Fig. 2).

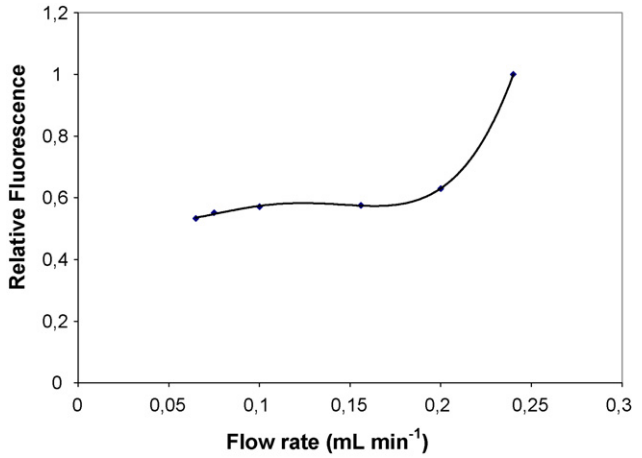

Fig. 2. Influence of flow rate on eo retention. Membrane of nylon; eo concentration 1×10^{-7} mol L^{-1}.

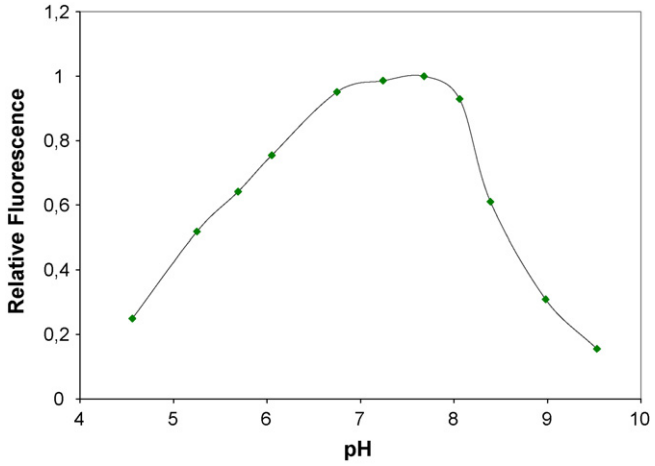

Fig. 3. Influence of pH on nickel retention. Membrane of nylon; eo concentration 1×10^{-7} mol L^{-1}; Ni(II) 29 ng L^{-1}.

3.5. pH of retention. Buffer concentration

The pH value of aqueous systems containing constant concentration of Ni(II), was adjusted between 4.5 and 9.5, by the addition of Tris buffer solution. The obtained results showed a maximum level of retention of Ni(II)-eo association for pH values of 6.75–8.00 (Fig. 3). For following experiences, a pH value of 7.00 was chosen, value close to the physiological.

Then, the buffer concentration was tested in order to obtain the maximum fluorescent signal. The concentration of Tris buffer was varied from 4×10^{-2}–0.5 mol L^{-1}. Buffer concentration of 1.1×10^{-2} mol L^{-1} was chosen as optimal.

3.6. Analytical parameters of developed methodology

Table 2 summarizes the studied experimental variables, the optimal values for separation/chemisorption of nickel on nylon membranes previously treated with eo dye and analytical parameters. The limit of detection (LOD) was calculated as $3s/m$, where s is the standard deviation of 10 successive means of the blank and m is the slope of the calibration curve (calibration sensitivity). The limit of quantification (LOQ) was calculated as $10s/m$. Range of linearity was evaluated by checking the linear regression coefficient (r^2) of the calibration curve. The linearity of the calibration curve was considered acceptable when $r^2 > 0.995$.

Table 2
Studied optimal experimental conditions and analytical parameters for nickel determination.

Parameters	Studied range	Optimal conditions
pH	5.5–9.5	7.0
Buffer Tris	4×10^{-2}–0.5 mol L^{-1}	1.1×10^{-2} mol L^{-1}
Eo concentration	1×10^{-9}–1×10^{-3} mol L^{-1}	1×10^{-7} mol L^{-1}
Contact time	0–700 min	5 min
LOD	–	0.13 ng L^{-1}
LOQ	–	0.44 ng L^{-1}
LOL	–	0.44–410 ng L^{-1}
Calibration sensitivity	–	6×10^{13} ng L^{-1}
r^2	–	0.999

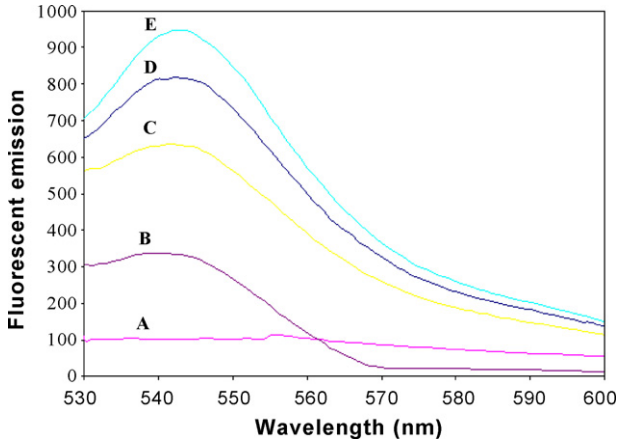

Fig. 5. Solid phase fluorescent emission of eo–Ni(II) system. (A) Membrane of nylon. (B) Membrane of nylon treated with eo 1×10^{-7} mol L^{-1}. (C) Idem B with Ni(II) 400 ng L^{-1}. (D) Idem B with Ni(II) 500 ng L^{-1}. (E) Idem B with Ni(II) 590 ng L^{-1}.

Fig. 4 shows SEM micrography of nylon membrane (A) and chemical structure of eo–Ni(II) association (B). Fig. 5 puts in evidence the enhancement of eo fluorescent emission caused by Ni(II) at increased concentrations. At optimal experiment conditions, quantitative retention was verified by a second filtration procedure through a new membrane; the obtained fluorescence signal was similar to eo signal.

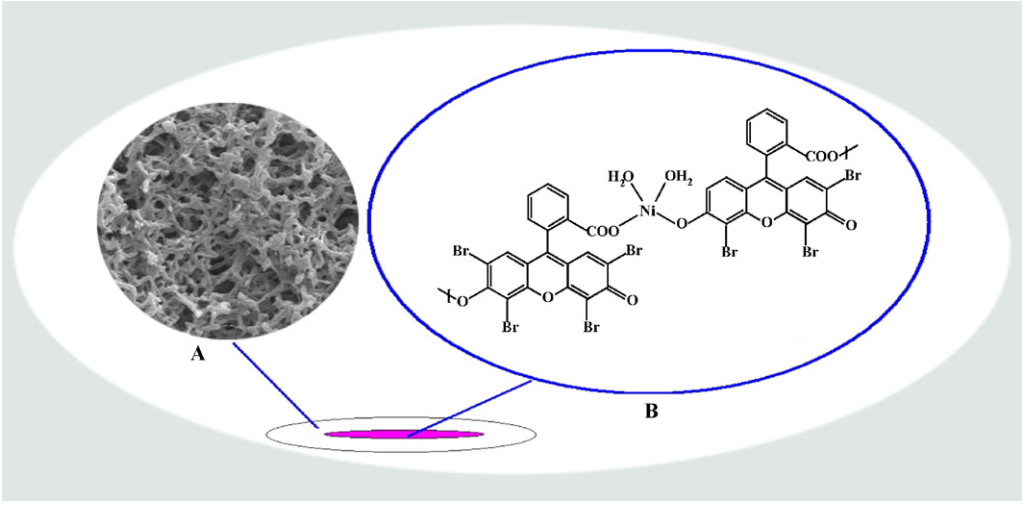

Fig. 4. Separation/chemisorption of nickel on nylon membranes treated with eosin dye. (A) SEM of nylon membrane and (B) eo–Ni(II) association.

4. Biological application

4.1. Studied subjects

Attending to smoking habits, the studied subjects can be described as follow:

Group 1: Non-smokers were selected as control constituted by 10 subjects.
Group 2: Passive smokers, second hand smoker (SHS), constituted by 9 subjects.
Group 3: Smokers of 3–5 cigarettes/day with 10 years old habit, constituted by 10 subjects.
Group 4: Smoker of 10 cigarettes/day with 10 years old habit, constituted by 7 subjects.
Group 5: Smokers of 20 cigarettes/day with 10 years old habit, constituted by 7 subjects.
Group 6: Smokers of 40 cigarettes/day with 10 years old habit, constituted by 5 subjects.
Group 7: Two non-smoker with tobacco chewing habit (TChH).

4.2. Biological sampling

In order to assure the obtention of representative samples, subjects received detailed information about the collection protocol.

For preventing subsequent urine interferences, subjects were instructed as follow:

- Do not intake vitamin or mineral aggregated 36 h before urine collection.
- Do not drink tap water during 24 h previous to sample collection.
- The first morning urine sample is preferred as sample, collecting the medium fraction.
- Samples must be directly remitted to laboratory for analysis; if it is not possible, they must be preserved at 277 K until analysis.

4.3. Physical and chemical characterization of urine samples

Once in the laboratory, urine samples were observed and characterized respect to physical appearance in order to stablish variables that could interfere in Ni(II) determinations. All processed samples can be namely considered within the normal physical parameters.

Samples were centrifuged for 10 min at $1000 \times g$. Supernants were reserved for nickel examination. Urine samples were tested using commercial reagent strips and clinical parameters pH, urobilinogen, glucose, ketones, bilirubin, proteins, nitrite, blood, specific gravity and leucocytesin were determined. Processed samples can be mainly considered within the normal clinical parameters.

Urine sediments were observed using optical microscope and classified with the intention of verifying the existence of some correlation between type of sediment and tobacco addiction level. The more frequents sediments were leukocites, piocites, hematies, normal cells, mucus, bacterias, and crystal of different types (urates, uric acid and oxalates).

4.4. Stability studies of urine samples

Biological samples were spiked with increasing amounts of Ni(II) and were processed by proposed methodology at different times during 1 month with preservation in refrigerator. Obtained results showed good precision with CV (variance coefficient) defined as s/x_{medium}, between 0.01 and 0.03; it can be inferred that during the studied period, urinary nickel shown optimal stability.

4.5. Dilution test

In order to stablish the proper volume of each urine sample for realizing Ni(II) determination, several sample volumes were assayed. The adequate dilution for each sample was that signal which intensities fall into the linearity range of the developed methodology. Dilution test was of 5000 μL for subjects with minor exposition and of 0.125 μL for the most exposed subjects. These dilution factors were adopted for the following studies.

Nickel contents were determined by the proposed methodology, employing the obtained volume samples through test dilution.

5. Tolerance studies

The effect of foreign ions on the recovery of Ni(II) was tested. An ion was considered as interferent, when it caused a variation in the fluorescent signal of the sample greater than ±5%. Table 3 shows the obtained results for assayed ions. For the most part of cations and anions studied an excellent tolerance was obtained for interferent/Ni(II) 1000/1 ratio, at optimal working conditions. Cd^{2+}, Cr^{3+}, Mg^{2+}, Mn^{2+}, Al^{3+}, Cu^{2+} and Co^{2+} are good tolered as many as interferent/Ni(II) 100/1 ratio.

Urine samples were tested using the developed methodology with good results; this fact put in evidence the versatility of the new methodology for the quantification of nickel traces in this biological matrix.

6. Analytical performance

The accuracy of the methodology was performed using the standard addition method. The reproducibility of the method was evaluated repeating 6 times the proposed approach for each addition in a total of three level of spiked Ni(II) for each sample. The recoveries of Ni(II) in 7 urine samples of 50 studied, based on the average of replicate measurements, are illustrated in Table 4; the

Table 3
Study of interferences.

Ion	Tolerated interferent/Ni(II) ratio	Fluorescent emission	%RE[a]
CO_3^{2-}	1000/1	557.371	0.51
SO_4^{2-}	1000/1	548.397	−1.10
NO_3^-	1000/1	550.383	−0.75
CH_3COO^-	1000/1	548.431	−1.10
Cl^-	1000/1	562.484	1.43
Br^-	1000/1	561.325	1.22
I^-	1000/1	558.170	0.65
F^-	1000/1	561.253	1.21
K^+	1000/1	557.537	0.54
Na^+	1000/1	555.820	0.23
Zn^{2+}	1000/1	563.582	1.63
Fe^{3+}	1000/1	548.276	−1.13
Ca^{2+}	1000/1	559.995	0.98
Bi^{3+}	1000/1	557.371	0.50
Ba^{2+}	1000/1	548.397	−1.10
Sr^{2+}	1000/1	550.383	−0.75
Pb^{2+}	1000/1	548.431	−1.10
Cd^{2+}	100/1	562.584	1.45
Cr^{3+}	100/1	561.325	1.22
Mg^{2+}	100/1	558.170	0.65
Mn^{2+}	100/1	561.250	1.20
Al^{3+}	100/1	546.890	−1.38
Cu^{2+}	100/1	555.762	0.22
Co^{2+}	100/1	560.053	0.99

[a] Percent relative error.

Table 4
Urinary nickel determination of subjects ordered by growing tobacco addiction level. Recovery study.

Sample[a]	Ni(II) added ($ng\,L^{-1}$)	Ni(II) found ± CV ($ng\,L^{-1}$)	Recovery (%, $n=6$)
1	–	13 ± 0.02	–
	40	53 ± 0.03	100.00
	180	200 ± 0.02	103.80
	290	300 ± 0.04	98.96
13	–	135 ± 0.04	–
	60	196 ± 0.04	101.66
	180	317 ± 0.09	101.11
	300	433 ± 0.06	99.33
25	–	564 ± 0.06	–
	180	745 ± 0.04	100.55
	300	866 ± 0.01	100.66
	590	1155 ± 0.02	100.17
33	–	1021 ± 0.02	–
	180	1201 ± 0.02	100.00
	300	1322 ± 0.03	100.33
	590	1620 ± 0.03	101.53
39	–	1934 ± 0.03	–
	060	1995 ± 0.01	101.67
	180	2111 ± 0.06	98.33
	300	2235 ± 0.04	100.33
46	–	3743 ± 0.01	–
	180	3926 ± 0.08	101.67
	300	4045 ± 0.07	100.66
	590	4338 ± 0.04	100.85
50	–	4443 ± 0.07	–
	180	4623 ± 0.02	100.00
	300	4750 ± 0.01	102.33
	590	5039 ± 0.06	101.02

[a] Only 1 urine sample/group has been informed.

obtained results showed that the proposed method is suitable for the determination of Ni(II) in such biological samples, for all range of studied concentrations. The obtained results were good correlated with the total number of smoked cigarettes of each studied subjects (Fig. 6).

In order to determine the repeatability (within-day precision) of the method, urine replicate samples ($n=6$) were analyzed by proposed methodology. The precision was better than 0.02 CV for urinary nickel. The reproducibility (between-day precision) was also evaluated over 3 days by performing six determinations each day with a CV of 0.052. The different groups were evaluated using one-way analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparison test with satisfactory results.

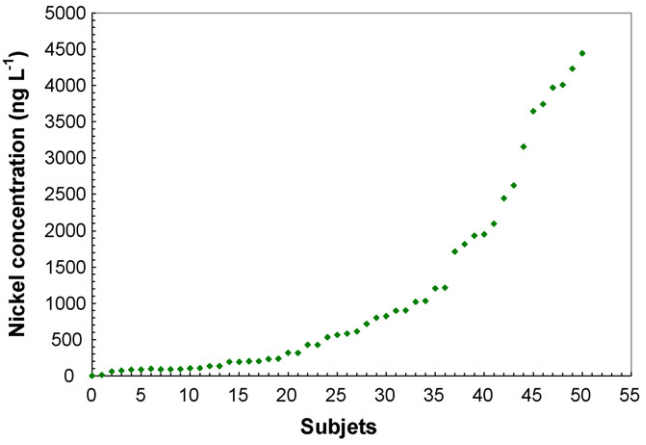

Fig. 6. Correlation of nickel contents with tobacco addiction level.

7. Conclusions

Nickel chemisorption has been proposed for separation/preconcentration on nylon membranes containing eo dye. The developed methodology is very simple, friendly environmental, inexpensive and fast. The reached sensitivity was comparable at those of atomic spectroscopies. High selectivity was evidenced by good tolerance at elevated levels of regular foreign constituents. Precision and accuracy were tested with excellent results. In addition, stability of urine samples during a month was studied and tobacco addiction level of 50 subjects was satisfactory correlated with found nickel contents. The proposed methodology represents an alternative to the routine metal analysis methods, with the advantage of using a simple and inexpensive instrumental. Attending to crescent urinary nickel concentration associated to tobacco addiction and the subsequent risks on health, efforts should be made by the control agencies and health agents at discourage the consumption of cigarettes and the tobacco chewing habit.

Acknowledgements

The authors wish to thank LABMEB (National University of San Luis) for microscopy studies, Instituto de Química San Luis - Consejo Nacional de Investigaciones Científicas y Tecnológicas (INQUISAL-CONICET), FONCYT (Fondo Nacional de Ciencia y Tecnología), and National University of San Luis (Project 22/Q828) for the financial support.

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Glossary

eo: eosin.
LOD: limit of detection.
LOQ: limit of quantification.
LOL: limit of linearity.
%CV: % coefficient of variance.
ICP-MS: inductively coupled plasma mass spectrometry.
ETAAS: electrothermal atomic absorption spectroscopy.
SHS: second hand smoker.
TChH: tobacco chewing habit.