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Natural zeolites for pharmaceutical formulations: Preparation and evaluation of a clinoptilolite-based material

Guido Cerri [a, *], Mauro Farina [a], Antonio Brundu [a], Aleksandra Daković [b], Paolo Giunchedi [c], Elisabetta Gavini [c], Giovanna Rassu [c]

[a] Department of Natural and Territorial Sciences, University of Sassari, Via Piandanna 4, 07100 Sassari, Italy
[b] Institute for the Technology of Nuclear and Other Mineral Raw Materials, Bulevar Franš d'Eperea 86, 11000 Belgrade, Serbia
[c] Department of Chemistry and Pharmacy, University of Sassari, Via Muroni 23/a, 07100 Sassari, Italy

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ABSTRACT

Aim of this work was the preparation, starting from a clinoptilolite-rich rock, of a material suitable for the development of pharmaceuticals. In particular, the purpose was to obtain a reproducible product that maximizes zeolite properties and minimizes any kind of interference chemical, mineralogical and microbiological. In evaluating the material for the planned use, the recommendations and procedures of European, US and Japanese Pharmacopoeias were taken as benchmark to the largest extent possible. A set of technological properties was also determined.

The prepared material, containing ≈90 wt.% of Na-clinoptilolite, was obtained through a replicable process, and do not contains fibrous minerals classified as carcinogenic by the IARC. Chemical analyses evidenced contents of trace metals below the more restrictive limits established by *Eur Ph.*, USP and JP for "bentonite" – taken as reference due to the similarities between smectites and zeolites, and because of the lack of a Monograph on clinoptilolite. The oral bioaccessibility of potential harmful elements, tested simulating the transit in the gastrointestinal tract according to *Eur Ph.*, was three to six orders of magnitude lower than the permissible daily exposure established by USP. The microbiological quality of the material complied with the acceptance criteria of *Eur Ph.* The clinoptilolite structure was not significantly affected by sterilization process nor by simulated gastric juices.

As concerns the characteristics determined, the prepared material is suitable for the development of systems exploiting clinoptilolite's properties as pharmaceutical excipient.

1. Introduction

Zeolites and clays share some properties (e.g., cation exchange capacity, reversible dehydration) although their structures are clearly different, being respectively tectosilicates and phyllosilicates. Natural clays are widely used in pharmaceutical industry, both as excipients and active agents [1–3], and required specifications are reported in Monographs of international Pharmacopoeias – e.g., *Eur Ph.*, USP, JP [4]. Despite the similarities between some clays, in particular smectites, and zeolites [5], and notwithstanding the growing interest of studying the zeolites for biomedical applications, reported in several reviews [6–11], natural zeolites as well as their required characteristics are not yet specified in the aforementioned Pharmacopoeias nor in the *Inactive Ingredients Guide for Approved Drug Products* [12]. Zeolites, both natural and synthetic, were investigated as drug carriers, adjuvants in anticancer therapy, dietetic supplements or antimicrobial agents [6,7,13]. As concerns these applications, clinoptilolite is the most studied among natural zeolites [8–10], and also different surfactant-modified forms were evaluated as carriers for slow release of some drugs [11,14,15]. Clinoptilolite has the same framework topology of heulandite, with two channels parallel to the *c*-axis (7.5 × 3.1 and 4.6 × 3.6 Å in width), and two additional channels, both 4.7 × 2.8 Å, parallel to [100] and [102] [16]. Deposits of clinoptilolite are distributed worldwide; in Italy, outcrops of potential economic interest are located in Sardinia [17]. Clinoptilolite was not classified as carcinogenic to humans [18]. This zeolite turned out not significantly toxic in oral or parenteral toxicity studies in animals [19], and its ingestion is well tolerated [20,21]. Clinoptilolite-based anti-diarrheic and antacid formulations for

* Corresponding author. Tel.: +39 079228621; fax: +39 079233600.
E-mail address: gcerri@uniss.it (G. Cerri).

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humans were commercialized [22,23], and this zeolite is the main constituent in a large number of currently marketed dietary supplements [8,24]. Due to the high resistance in acid media, together with the high selectivity toward some cations, clinoptilolite was used to prepare chocolate and biscuits for cesium decontamination of children after the Chernobyl disaster [16]. The possibility to detoxify the organism of small mammals contaminated with lead, by using clinoptilolite as food supplement, was demonstrated [25], and an increasing number of patents and products claims the use of clinoptilolite as an agent that, once ingested, is capable to reduce toxic substances in the human body [8,24,26,27], or is able to relieve the symptoms of any gastrointestinal irritation induced by whatever substance [28,29]. At the same time it should be noted that, among the numerous publications dealing with medical applications of zeolites, a frequent drawback is represented by an inadequate or incomplete characterization of the mineral matter [9]; in particular, quite often a zeolitized rock is considered as zeolite, disregarding the presence of other phases. Furthermore, some papers presenting results of experiments performed on humans do not report a characterization of the clinoptilolite-based material used in these studies [27,29]. As pointed out by Ferdov et al. [13], the natural origin of clinoptilolite presumes a chemical composition determined by the specific conditions of the deposit. Consequently, chemical and mineralogical characterization of material, performed with adequate techniques, is imperative. Negative biological effects may arise because of a high content of heavy metals in a zeolitized rock [30]. Minerals associated to natural zeolites might interfere, develop adverse reactions, or simply reduce, by "dilution" effect, the expected activity of the zeolite. Moreover, the possible presence of bacteria, mold or yeast should be considered.

This study represents the first step of a research – supported by the Italian Ministry for Education, University and Research – aimed to develop pharmaceuticals based on natural zeolites for oral administration of drugs. A first aspect must be studied: the possibility to obtain, starting from a clinoptilolite-rich rock, a reproducible material that maximizes zeolite properties and minimizes any kind of possible interference (chemical, mineralogical and microbiological). In detail the purpose of the present work is to investigate *i)* the possibility to prepare a reproducible and near-pure clinoptilolite-based material; *ii)* the content of potential harmful minerals and elements in the material prepared; *iii)* the oral bioaccessibility of the elements, determined in simulated gastrointestinal environment; *iv)* the presence of bacteria, mold or yeast in the material and the efficacy of a sterilization process; *v)* the effects of the simulated gastric juices and of the sterilization process on the clinoptilolite structure; *vi)* a set of technological properties (cation exchange capacity, specific surface area, porosity, zeta potential, point of zero charge, water uptake, pH of a given suspension, particle size distribution, powder flowability, true and tapped density), in view of the pharmaceutical application. In the evaluation of the material, the recommendations and procedures of the European and United States Pharmacopoeias are taken as benchmark as much as possible. When feasible, the results are compared with the specifications for "bentonite", the excipient closest to clinoptilolite already classified by the *Eur Ph.*, USP and JP.

2. Experimental

2.1. Material

A clinoptilolite-rich epiclastite, already successfully used in the past to develop a topical application (sample "LacBen" in Refs. [31,32]), was used as starting material. The rock, Oligo-Aquitanian in age, was sampled at Bortivuile locality, Sassari province, Sardinia island, Italy (DMS coordinates: 40°25′8.548″N; 9°4′15.035″E). The mineralogical composition, determined by Cerri et al. [31], is: clinoptilolite 66 ± 4 wt.%; feldspars 18 ± 2 wt.%; opal-CT 13 ± 1 wt.%; quartz 3 ± 1 wt.%; traces of biotite.

2.2. Beneficiation process

The rock was granulated to a size < 2.5 cm using a steel jaw crusher. The granules were then submitted to an autogenous comminution in a Retsch planetary mill (agate jars) without grinding media, to get mainly an abrasion of clinoptilolite microcrystals (smaller and softer than coexisting feldspars and quartz), instead of a size reduction of all phases present in the rock. Two comminution cycles (15 min each) at 70 rpm were performed on approximately 200 g of granulated material. At the end of each cycle, the powder was sieved for 15 min using an automatic siever (Controls D407) to recover the fraction <125 μm. After each cycle, the class >125 μm was discarded. The process was repeated until about 250 g of fraction <125 μm was obtained. To get monomineralic particles, approximately 11 g of the powder was dispersed in 500 ml of deionized water, and sonicated for 5 min (ultrasonic bath Sonorex Super RK 106). The supernatant was placed in a 2-l beaker, whereas the settled fraction was mixed with fresh deionized water and sonicated for another 5 min. After four sonication cycles, the total initial amount (11 g) was placed in the 2-l beaker (water column: 16 cm), stirred, then left to settle for 100 h. Thereafter, the supernatant was eliminated, to reduce possible contaminants like soluble salts and clays. The sediment was then suspended once again (water column: 8 cm) and left to settle for 1 min, to eliminate the coarser fraction, containing concentrates of quartz and feldspars. The supernatant was recovered and dried in a ventilated oven at 40 °C. About 175 g of enriched material, divided in 8 lots, was prepared. Each lot was analyzed by quantitative X-ray diffraction (Section 2.4), in order to evaluate the reproducibility of the enrichment process. Finally, the lots with homogenous composition were mixed together (V-mixer ARTHA AISI 304, constant speed of 18 rpm) and used in further experiments (sample FA), whereas the others were discarded.

2.3. Preparation of the Na-clinoptilolite

Clinoptilolite was conducted in Na-form to get a material chemically more homogenous and easier to reproduce, and to reduce the content of heavy metals that may be present as exchangeable cations. The enriched powder was contacted (solid/liquid = 50 g/l) with a 1 M NaCl solution (VWR, *Eur Ph.* grade; purity 99.9%), by performing 11 exchange cycles, 2 h each, at 65 °C and under continuous stirring. The powder was then rinsed with deionized water, until chloride were no longer detected (test with $AgNO_3$), and dried in oven at 40 °C. The final material (denoted as FA-Na) was rehydrated for 24 h (22 ± 1 °C, 53 ± 2% of relative humidity, monitored with an EBRO EBI-TH1) in a desiccator containing a saturated solution of $Ca(NO_3)_2$.

2.4. X-ray diffraction (XRD)

X-ray analyses were performed using a Bruker D2-Phaser diffractometer under the following conditions: 30 kV, 10 mA, CuKα radiation, LynxEye detector with an angular opening of 5°, 2θ range 5.8–70°, step size 0.020°, time per step 2 s, spinner 15 rpm. Quantitative mineralogical compositions, was obtained by applying the Rietveld Method (software Bruker Topas 4.2). Samples were micronized (McCrone mill) after mixing with 20 wt.% of α-Al_2O_3 (Buehler) as internal standard. The powders subjected to the tests

described in the Sections 2.8 and 2.10 were analyzed, in duplicate, to compare their X-ray patterns with that of FA-Na sample.

2.5. Cation exchange capacity (CEC)

For determination of cation exchange capacity (CEC) of FA-Na, material was dispersed (2 g in 67 ml) in a 0.5 M NH_4Cl solution (Sigma–Aldrich, purity ≥ 99.5%), kept at 65 °C under continuous stirring. Duration of the five successive exchange cycles were: 30 min; 30 min; 1 h; 2 h; 2 h. The concentrations of released exchangeable cations (Na^+, K^+, Ca^{2+} and Mg^{2+}) were determined by atomic absorption spectrophotometry (AAS) by using Analytic Jena Spekol 300. Cation exchange capacity (CEC) was calculated as the sum of released cations.

2.6. Thermal analyses

Thermogravimetric and Differential Thermal Analyses (TG–DTA) of FA-Na were performed (in duplicate) with a TA-Instrument Q600. About 20 mg of sample were heated up to 900 °C (10 °C/min; alumina crucibles; static air atmosphere; calcined Al_2O_3 powder as reference). Results were evaluated with the software TA-Universal Analysis.

2.7. Chemical analyses

Chemical analysis of FA-Na was executed in a certified laboratory (Actlabs, Ancaster – ON, Canada), where the sample was prepared through the routinely procedure (pulverization with mild steel mill). Major elements were determined by using Induced Coupled Plasma – Optical Emission Spectroscopy (ICP-OES) after lithium metaborate/tetraborate fusion and HNO_3 digestion. Iron (Fe^{2+}) was measured by the titration method. Trace elements, except when specified, were determined by ICP-OES and/or ICP-Mass Spectrometry (MS), after total digestion of the sample with four acids (HCl, HNO_3, $HClO_4$, and HF). Elements – Au, Br, Cl and Ir were determined by Instrumental Neutron Activation Analysis (INAA). Fluorine (F) was analyzed by Ion-Selective Electrode (ISE) after sample fusion. Total N was determined through Total Kjeldahl Nitrogen (TKN) method. Carbon (C-total and C-organic) was analyzed by Infrared Spectroscopy (IR). Since ActLabs warns that total digestion might not be very effective for the dissolution of As, Ce, Cr, Eu, Hf, La, Lu, Nd, Sb, Sc, Sm, Ta, Tb, U, and Yb, these elements were determined also by INAA. To verify possible contamination caused by different grinding media, heavy metals were determined by ICP-OES and MS also on two FA-Na samples pulverized in our laboratories by using an agate mortar and a Retsch MM400 equipped with zirconia cups and balls.

2.8. Digestive simulation and resistance in gastric environment

Three tests were performed, using the procedures described in the *Eur Ph.* [33], to simulate the transit of FA-Na in the stomach (*Test 1* and *Test AGJ*) and in the whole gastro-intestinal tract (*Test 2*). The tests were conducted to measure the release of elements (in particular, heavy metals) from FA-Na, and to verify the resistance of clinoptilolite. A dissolution Apparatus 2 (Teflon-lined paddle), required for dissolution test for solid dosage form, was used, set at 37 ± 0.5 °C and 100 rpm. All solutions were prepared with Milli-Q water.

Procedures

Test 1: 0.75 g of FA-Na were mixed with 750 ml of a 0.1 M HCl solution (pH = 0.94) for 120 min.

Test 2: the first stage was the same as in *Test 1*, then 250 ml of a 0.2 M Na_3PO_4 solution was added to the mixture (pH increased to 6.8) and the suspension was stirred for 240 min.

Test AGJ: procedure was the same as in *Test 1*, but using Artificial Gastric Juice (AGJ) containing pepsin [34].

After these tests, the powders were analyzed by XRD (Section 2.4). Blank solutions and elutes were analyzed by ICP-MS/OES at Actlabs (Section 2.7); the results of *Test 2* were reported to a solid/liquid ratio of 1 g/l. The pH was measured at the beginning and at the end of the tests.

2.9. Total aerobic microbial count (TAMC) and total yeast and mold count (TYMC)

TAMC and TYMC were evaluated according to the *Eur Ph.* [35]. Sample FA-Na was dispersed in peptone water (Oxoid) adjusted to pH 7.2 (1 g in 9 ml = 1:10 dilution); dilutions of 10^2, 10^3 and 10^4 were also prepared. Tryptone soya agar (Oxoid) and sabouraud dextrose agar (Oxoid) were sterilized, cooled to 45 °C, and used as culture media. Then, 1 ml of each dilution was poured in sterilized Petri dishes, and 20 ml of a culture media was added to each plate. Plates containing sabouraud dextrose agar were incubated at 20 °C for 7 days, while those with tryptone soya agar were incubated at 37 °C for 5 days. All tests were made in triplicate; the average of the counted colonies was calculated, multiplied by the dilution factor and expressed as number of microorganism per gram of sample.

2.10. Sterilization process and sterility test

Sterilization was performed by heating FA-Na powder at 160 °C for 3 h (*Eur Ph.* [36] requires t ≥ 2 h). A heated sample was analyzed by XRD (Section 2.4). Sterility test was carried out according to *Eur Ph.* [35]. Tryptone soya broth (Oxoid) and thioglycollate USP (Oxoid) were used as culture media, and sample concentration was 4 g/l. The inoculated media were incubated for 14 days, then 1 ml was transferred to fresh vessels of the same medium and incubated for another 5 days. Finally, bacterial growth was checked.

2.11. Surface area and porosity

Adsorption–desorption isotherms were obtained by nitrogen adsorption at 77 K using a Sorptomatic 1990 Thermo Finnigan device. Prior to adsorption, FA-Na powder was degassed first for 4 h at room temperature under vacuum and then for 24 h at 383 K at the same residual pressure. The resulting isotherms were analyzed by Software ADP Version 5.13 Thermo Electron. Total pore volume (V_{tot}) was obtained by applying Gurevitsch's rule at a relative pressure $p/p_0 = 0.98$. The specific surface area (S_{BET}) was determined according to the Brunauer, Emmett, Teller method. Micropore and mesopore volumes (V_{mic} and V_{meso}) were obtained by applying the Dubinin–Radushkevich equation and the Dollimore and Heal method, respectively.

2.12. Zeta potential and point of zero charge

The zeta potential was measured using a Zetasizer NanoZS90 (Malvern Instruments Ltd., UK). Aqueous suspensions (0.5 mg/ml) of FA-Na were dispersed using an ultrasonic bath and an average of 6 measurements was taken to represent the measured potential. Prior to the measurements, the operating conditions were proved and adjusted using a calibrated latex dispersion supplied by the manufacturer (zeta potential −50 ± 5 mV). Zeta potential was measured both in distillated water at pH 6.05, and in HCl solutions at different pH values.

The point of zero charge (pH_{pzc}) was determined by the batch equilibrium method using KNO_3 (0.001, 0.01 and 0.1 mol/l) as a background electrolyte. The initial pH value (pH_i) of the solution was adjusted by small additions of 0.1 mol/l HNO_3 or 0.1 mol/l KOH. In the initial solution (25 ml), 0.05 g of FA-Na was added and the bottles were shaken for 24 h at room temperature, centrifuged at 10,000 rpm for 10 min and the pH value of supernatants determined (pH_f).

2.13. SEM observations

Morphological observations were carried out on the unprocessed rock (sample "LacBen") and FA-Na. The samples, placed on aluminum stubs, were gold coated (AGAR automatic sputter coater B7341) and observed using a ZEISS EVO LS-10 Scanning Electron Microscope.

2.14. Technological characterizations

The water uptake capability of FA-Na was determined with a modified Enslin apparatus as described by Rassu et al. [37]. The water adsorbed by capillarity was measured at predetermined times (15, 30, 60, 120, 180, 300 s). The test was performed at 20 ± 1 °C and 44 ± 8% RH, using Milli-Q water. The pH of a given suspension of FA-Na was measured following the methodology described for clay–water suspensions [38], by suspending 4 g of FA-Na in 200 ml of water. The pH was measured after 2 min of continuous stirring. The fundamental and derivates properties of FA-Na powder, useful for its application in the pharmaceutical field, were determined according to the Pharmaceutical Technical Procedures of the *Eur Ph.* [33]. All determinations hereafter described were performed in triplicate. Particles size was determined by light diffraction using a Coulter Laser Sizer LS 100Q, and the uniformity of the distribution was expressed by the SPAN Index = $(d_{90} - d_{10})/d_{50}$, being d_{xx} the diameter at the 90th, 10th and 50th percentile. The true density of the powder (D_t) was measured using a helium pycnometer AccuPyc 1340. The "apparent volume" V_0 (i.e., volume of a given mass m of powder introduced in a graduated cylinder without any compacting) and the "tapped density" of the powder (i.e., m/V_{1250}, where V_{1250} is the volume after 1250 standardized taps) were also determined. To verify if V_{1250} was already an asymptotic value, also V_{2500} was determined. The flowability of the powder was dynamically measured using a Flowability Tester BEP2 (Copley Scientific). The Hausner Ratio ($HR = V_0/V_{1250}$) and the Compressibility Index ($CI = 100 \times [(V_0 - V_{1250})/V_0]$), used to predict powder flow characteristics, were calculated and compared with the scale of flowability given in the *Eur Ph.* [33].

3. Results and discussion

At the end of beneficiation process, eight lots of enriched material, having a mass between 22.8 and 28.5 g and a clinoptilolite content from 89.2 ± 2.0 to 91.7 ± 2.0 wt.%, were obtained. Seven lots were very homogenous, with a zeolite content between 90.3 ± 2.0 and 91.7 ± 2.0 wt.%. Once mixed, these seven lots (about 150 g) evidenced the mineralogical composition given in Table 1 (sample FA). Notably, the clinoptilolite content (90.2 ± 2.0 wt.%) is slightly higher than in other ingestible zeolite-based drugs already commercialized [22,23] or in drugs under development [24,26]. Concerning the phases other than clinoptilolite (Table 1), the prepared material contains less "impurities" (i.e., associated minerals) than an Italian smectite currently employed in a drug for gastrointestinal diseases [39], as well as of the aforementioned zeolite-based drugs. It is noteworthy the very low quartz content (0.4 wt.%) and the absence of zeolites recognized as carcinogenic (erionite – [40]), or cytotoxic (mordenite – [41]), not detected in sample FA-Na by XRD nor SEM investigations.

The beneficiation process, developed from method described by Brundu et al. [42], showed reproducibility and effectiveness, increasing the clinoptilolite content from 66 to 90 wt.%. Furthermore, is a low-cost method, and does not involve the use of harmful media (e.g., heavy liquids) unacceptable in the pharmaceutical sector. Conceptually the technique is similar to those industrially employed to process clays, and also the purity attained is comparable [43], indeed traces of quartz and opal-CT are still present, for example, in a commercially available montmorillonite used in food packaging [44].

The cation exchange capacity (CEC) of FA-Na, the sodium form of enriched material, corresponds to 2.33 meq/g (Table 2). The amount of Na^+ released reached 93.6% of the CEC, demonstrating that a near homoionic Na-clinoptilolite was obtained. The obtained high CEC was due to the high clinoptilolite content in FA-Na and specific conditions for sodium pretreatment [45]. The majority of investigated biomedical applications of zeolites is based on their cation exchange ability, that's why a high CEC is important. Na-form offers some advantages, because sodium is an essential element well tolerated by human organism [46], and is easily adsorbed and released by clinoptilolite [47]. Furthermore, the preparation of a Na-clinoptilolite allows to get a material chemically more homogenous and easier to reproduce, and should help to reduce the content of heavy metals that may be present as exchangeable cations.

The TG, DTG and DTA paths of FA-Na, in Fig. 1, are consistent with the data reported by Sternik et al. [48] for a Na-clinoptilolite.

The chemical analyses of FA-Na are reported in Table 3a and b, compared to the composition of the bulk rock [31]. As far as major elements (Table 3a), the reduction of iron content is mainly related to the beneficiation process, whereas the variations of sodium, potassium, calcium and magnesium are fundamentally due to the Na-exchange. A positive consequence of the beneficiation and sodium exchange processes, is clearly evident from the data in Table 3b, indeed the content of trace elements in FA-Na is always lower than that in the starting rock (with the only exception of Cr), in particular for Ba, Cs, Cu, Pb, Rb, Sr, Th, U, V and Zn. Total and organic C, Cl, F, Hg, N and Fe^{2+} are below the detecting limits. The elements in Table 3b include those elements traditionally considered as toxic or potentially dangerous for human health [4], unfortunately, among zeolite-based pharmaceutical products, only those employing Cuban zeolites report the content of some of these elements [22–24]. Compared to the Cuban materials, recently reported by Selvam et al. [24], FA-Na shows lower amounts of Ba, Cu, Mn, S and Zn and higher values of Pb and Cr; as far as the major elements, there is a marked discrepancy in the iron content, lower

Table 1
Mineralogical composition of sample FA, the prepared clinoptilolite-rich powder (wt.%, e.s.d. = estimated standard deviation).

	Clinoptilolite	Quartz	Biotite	Feldspars	Opal-CT	Amorphous
Content	90.2	0.4	1.2	3.2	1.2	3.8
e.s.d.	±2.0	±0.1	±0.2	±0.3	±0.2	±1.0

Table 2
CEC determination: cations released from FA-Na in a 0.5 M NH_4Cl solution.

	Na^+	K^+	Ca^{2+}	Mg^{2+}	TOT
meq/g	2.18	0.03	0.02	0.10	2.33
e.s.d.	±0.02	±0.01	±0.01	±0.01	±0.05

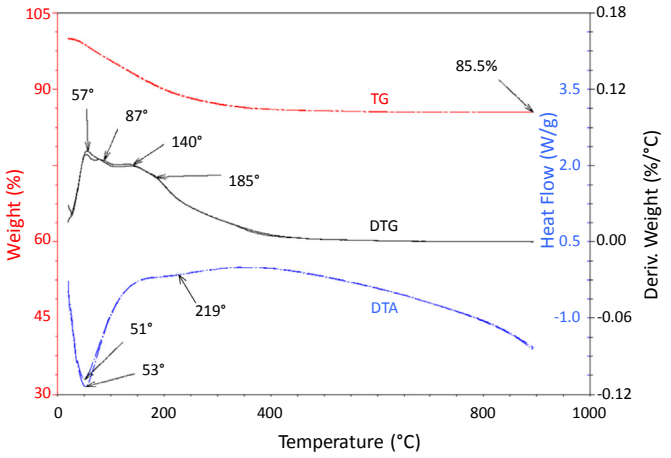

Fig. 1. TG–DTG–DTA of FA-Na.

in FA-Na (0.5 vs. 1.8–2%), whereas the Na-exchange is the main reason of the differences in alkaline and alkaline-earth elements. A significant evaluation of FA-Na as a component for formulations to be orally administered, can be done by comparing its chemical composition with the data reported by Mascolo et al. [49] for a drug composed by 80% of smectite (montmorillonite) and 20% of sugar and vanillin, indeed the authors have considered almost all the elements listed in Table 3b. Basically, FA-Na and the smectite-based drug show comparable amounts of potentially harmful elements (hereafter, PHEs). Moreover, having in mind the limits reported for "bentonite" and "purified bentonite", the excipients closest to clinoptilolite included in International Pharmacopoeias, it can be noted that FA-Na satisfied even the more restrictive requirements: As $\leq$ 2 ppm (JP [50] – "bentonite"); Pb $\leq$ 15 ppm (USP [51] – "purified bentonite"). It must be underlined that the techniques used to analyze FA-Na are more accurate than the colorimetric test presently contemplated by *Eur Ph.*, USP and JP for the determination of lead (and heavy metals in general) in bentonite [38,51,52], a test considered qualitative rather than quantitative [53]. Basically, the analyses performed on FA-Na are in line with the procedures for the determination of elemental impurities recommended by USP<233> [54], that will become effective on December 1, 2015. The performed analysis by INAA, for the determination of elements for which "total digestion" procedure might be not very effective (Section 2.7), produced results superimposable to ICP-MS ones. Similarly, the use of agate or zirconia as milling media showed no significant differences, whereas mild steel caused a contamination, sometime strong, on a limited number of elements, indicated in Table 4.

Table 4
Contamination induced by mild steel grinding (ppm; compare with values in Table 3b).

	Co	Cr	Cu	Mn	Mo	Ni	Ta	W	Zr
ppm	77	42	12.2	58	2.4	123	1.9	10.9	151
e.s.d.	±2	±1	±0.2	±1	±0.1	±3	±0.1	±0.2	±3

If a zeolite is orally administered to take advantage of its CEC, the integrity of the structure in the gastrointestinal tract (GI-tract) is crucial. The Si/Al ratio strongly affects zeolite resistance [7], and clinoptilolite is generally recognized as acid-resistant, albeit acid leaching can determine dealumination and loss of crystallinity [55]. However, the Si/Al ratio in heulandite–clinoptilolite series may vary from 2.6 to 5.7 [56], an aspect that underlines the importance of material characterization. The structure of the clinoptilolite used in this study was not significantly affected by the digestive simulation tests (Fig. 2). The "digested" samples show X-ray patterns with a signal/noise ratio slightly lower than the initial one (FA-Na),

Table 3
Chemical composition of FA-Na compared to bulk rock (sample "*LacBen*", from Ref. [31] – values reported in brackets).

a) Major elements, in wt.%.

	SiO_2	Al_2O_3	Fe_2O_3	MnO	MgO	CaO	Na_2O	K_2O	TiO_2	P_2O_5	LOI	TOT
FA-Na	65.26	12.11	0.50	<0.01	0.51	0.35	5.85	0.43	0.18	0.05	14.76	100.01
e.s.d.	±0.09	±0.06	±0.02	–	±0.02	±0.01	±0.04	±0.02	±0.02	±0.01	±0.07	±0.36
LacBen	63.30	13.05	1.64	<0.03	1.40	2.69	1.76	1.64	0.26	0.08	14.05	99.87

b) Trace elements, in ppm. ICP-MS analyses, except [a](ICP-OES), [b](INAA), [c](IR), [d](ISE), [e](TKN) and [f](Titration).

	FA-Na	LacBen		FA-Na	LacBen		FA-Na	LacBen
Ag	0.18 ± 0.02	–	Gd	2.1 ± 0.2	2.7	Sb	0.2 ± 0.1	0.3
As	1.5 ± 0.1	1.7	Ge	<0.1	0.24	Sc[a]	3 ± 1	–
Au[b]	$<5\cdot10^{-3}$	–	Hf	4.2 ± 0.2	4.9	Se	0.7 ± 0.1	–
Ba	410 ± 10	499	Hg	$<1\cdot10^{-2}$	–	Sm	2.1 ± 0.1	3.4
Be	1.8 ± 0.1	2.7	Ho	0.4 ± 0.1	0.6	Sn	1.0 ± 0.1	2.1
Bi	0.11 ± 0.01	0.13	In	<0.1	<0.1	Sr	54.1 ± 2.4	340.2
Br[b]	<1	–	Ir[b]	$<5\cdot10^{-3}$	–	Ta	0.6 ± 0.1	1.0
C^c_{tot}	<10	–	La	16.1 ± 0.9	25.6	Tb	0.3 ± 0.1	0.4
C^c_{org}	<50	–	Li	3.1 ± 0.3	–	Te	<0.1	–
Cd	<0.1	<0.3	Lu	0.2 ± 0.1	0.3	Th	10.5 ± 0.3	15.5
Ce	29.4 ± 2.5	46.9	Mn	48 ± 2	–	Tl	<0.05	–
Cl[b]	20 ± 2	–	Mo	0.3 ± 0.1	0.4	Tm	0.2 ± 0.1	0.3
Co	0.8 ± 0.1	0.98	N[e]	<100	–	U	2.2 ± 0.1	4.2
Cr	8.2 ± 0.3	<5	Nb	5.3 ± 0.3	9.5	V	12 ± 1	21
Cs	0.89 ± 0.09	3.05	Nd	11.3 ± 0.7	18.1	W	0.2 ± 0.1	0.2
Cu	8.7 ± 1.4	14.6	Ni	3.2 ± 0.3	<5	Y	12.9 ± 0.4	15.0
Dy	2.0 ± 0.2	2.9	Pb	14.3 ± 0.2	23.4	Yb	1.2 ± 0.1	1.7
Er	1.2 ± 0.1	1.6	Pr	3.2 ± 0.2	5.3	Zn	19.6 ± 0.8	34.6
Eu	0.30 ± 0.04	0.52	Rb	23.2 ± 0.6	70.1	Zr	126 ± 3	165
F[d]	<10	–	Re	0.005 ± 0.001	–	FeO[f]	$<1\cdot10^2$	–
Ga	8.4 ± 1.2	13.5	S[a]	5 ± 1	–			

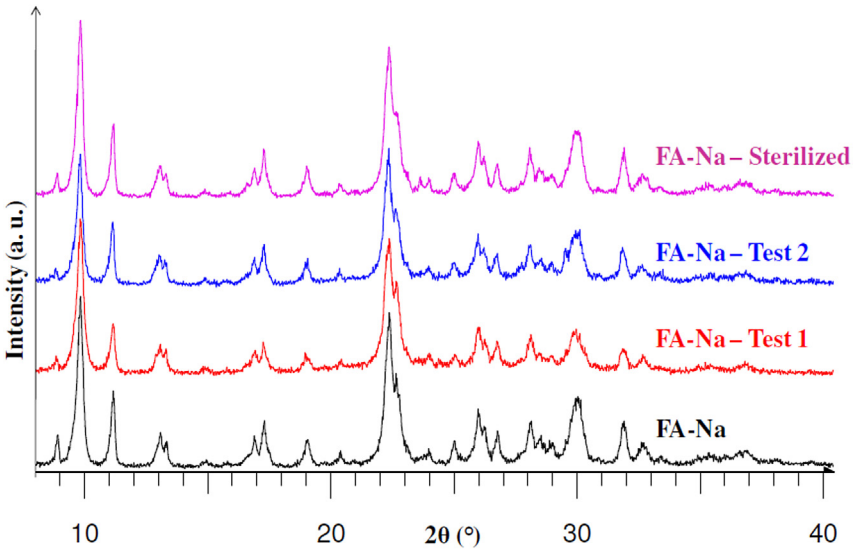

Fig. 2. XRD patterns of: FA-Na; FA-Na after the digestive simulation Tests 1 and 2; FA-Na after the sterilization process.

an effect only partially attributable to exchange phenomena that can modify the intensity of some peak [57], indeed a limited amorphization of the zeolite is testified by the small increase of the background (Fig. 2). The sample tested in Artificial Gastric Juice containing pepsin shows an X-ray pattern (omitted in Fig. 2) analogous to that of *Test 1*, confirming that enzymes did not increase the digestion of solid silicates [58].

The Environmental Health Criteria for clays used in pharmaceutical products recommend the determination of total content, mobility and bioavailability of PHEs [59]. These criteria are also reasonably applicable to zeolites employed, or evaluated, for medical purposes. It should be considered that even very low content of PHEs causes appreciable element increase into some organs [39]. The fraction of contaminant that is mobilized from a mineral phase(s) into the digestive juice is defined as the bioaccessible fraction [60]. This fraction is considered to represent the maximum amount of contaminant available for intestinal absorption, that's why the bioaccessibility is crucial in controlling the oral bioavailability of contaminants [61]. In-vitro gastrointestinal extraction, known as oral bioaccessibility, is important when assessing chemical risk to humans, and several procedures have been developed in attempts to mimic the effects of the digestion process [61,62]. However, in the evaluation of drugs and excipients the methods recommended by Official Pharmacopoeias (preferably, *Eur Ph.*, USP or JP) to simulate the transit in the GI-tract could be used. The release of elements from FA-Na in gastric (Test 1) and gastrointestinal (Test 2) environments, simulated according to *Eur Ph.* [33], are reported in Table 5. As expected, Na shows the highest release (38 ppm, i.e. 87.6% of the sodium amount in 1 g of FA-Na), basically referable to the exchange of sodium with hydrogen ions ($Na^+ \leftrightarrow H^+$), while release of Ca, Mg and K was very low in both tests. Concentration of Al measured after Test 1, indicates partial dealumination of clinoptilolite at pH 1 [55], however, when the pH increases during simulation of the passage in the intestine, precipitation of the major part of leached aluminum occurred. Similar behavior was noticed for iron, whereas the amounts of Si and Mn slightly increase from Test 1 to Test 2. These trends are expected because of the solubility/pH relationship of silica and Al^{3+}-, Fe^{3+}- and Mn^{2+}-hydroxides. Selvam et al. [24] evaluated the release of major elements from a clinoptilolite used in the manufacture of a product for oral ingestion, and found that Al release exceeds that of Si. The $Na^+ \leftrightarrow H^+$ exchange and the slight dealumination did not affect the pH during Test 1 (final pH = 0.96), because higher solid/liquid ratios are required to observe a variation [23].

The tests performed demonstrated that the oral bioaccessibility of minor elements contained in FA-Na is extremely low (Table 5). Values are always below the detection limits for Ag, Be, Bi, Cu, Hf, Hg, In, Li, Mo, Ni, S, Sn, Te, Tl, W and Zn. Furthermore, the values of Cr, Ge, Ho, Lu, Nb, Sb, Ta, Tm, V and Zr are close, or below, the limit of detection. The majority of the elements showed higher values in the first test, confirming that the main differences in results of bioaccessibility can be explained on the basis of the applied gastric pH [61]. Lead bioaccessibility was observed to be more dependent on stomach pH than As bioaccessibility, indeed when the acidic stomach environment is neutralized, Pb is largely removed from solution by precipitation and adsorption reactions, while As is not [62]. From this point of view, the behavior observed for Pb (that decreases from 3.8 to 0.3 ppb) and As (0.06 ppb in both tests) is typical. The CEC of clinoptilolite can contribute in variation of the bioaccessibility of some PHEs along the GI-tract, as observed by Danz et al. [26] for Pb and Hg. Similar behavior was noticed for barium, since it was initially released from zeolite (110 ppb in Test 1), then removed (<20 ppb in Test 2).

REE, Th, U and Y are usually found in phosphates like monazite, apatite, xenotime. These accessory minerals are typically present in traces, hence undetectable by XRD analyses. The variation of solubility with pH of these phosphates [63] might explain the higher release in Test 1 of the aforementioned elements; the simultaneous leaching of 50% of the phosphorus from FA-Na supports this hypothesis. In Test 2, the increase of pH and the availability of PO_4^{3-} (Section 2.8) favored the decrease of REE, Th, U and Y bioaccessibility.

There are no data in the literature on the oral bioaccessibility to humans of PHEs released from clinoptilolite. However, by reporting to a body weight of 80 kg the Reference Dose for Chronic Oral Exposure indicated in the IRIS database [64], it can be noted that the releases of the elements reported in Table 5 are at least two orders of magnitude below the reported limits.

The chemical compatibility of FA-Na for oral administration can be also assessed by comparing the releases of As, Cd, Cr, Cu, Hg, Mo, Ni, Pb and V with the permissible daily exposure, on a 50-kg person, established for these elements in the USP<232> [65]. By

Table 5
Elements released in media simulating the gastric (Test 1) and gastrointestinal (Test 2) environment (values in ppb; major elements listed first). [#]Not determined, because a Na_3PO_4 solution was used in Test 2. ICP-MS analyses, except for [a](ICP-OES).

Element	Test 1	Test 2	Element	Test 1	Test 2	Element	Test 1	Test 2
Na[a]	$38.0 \cdot 10^3$	n. d.[#]	Er	0.29	0.03	Sc	<10	19
Al[a]	$9.2 \cdot 10^3$	$0.4 \cdot 10^3$	Eu	0.09	<0.01	Se	2.4	<0.2
Si[a]	$3.1 \cdot 10^3$	$3.4 \cdot 10^3$	Ga	0.8	1.4	Sm	1.26	<0.01
K[a]	$1.0 \cdot 10^3$	$1.8 \cdot 10^3$	Gd	1.16	0.01	Sn	<1	<1
Mg[a]	$0.7 \cdot 10^3$	$1.0 \cdot 10^3$	Ge	<0.1	0.1	Sr	6.75	<0.04
Ca[a]	$0.4 \cdot 10^3$	$0.3 \cdot 10^3$	Hf	<0.01	<0.01	Ta	<0.01	0.01
Fe[a]	$2.2 \cdot 10^2$	$0.5 \cdot 10^2$	Hg	<2	<2	Tb	0.14	<0.01
P[a]	$1.4 \cdot 10^2$	n. d.[#]	Ho	0.12	<0.1	Te	<1	<1
S[a]	$<1 \cdot 10^3$	$<1 \cdot 10^3$	In	<0.1	<0.1	Th	0.59	0.07
Ag	<2	<2	La	7.46	0.14	Ti	7.0	4.3
As	0.06	0.06	Li	<10	<10	Tl	<0.01	<0.01
Ba[a]	110	<20	Lu	0.03	<0.01	Tm	0.04	<0.01
Be	<1	<1	Mn	1.9	3.0	U	0.08	0.06
Bi	<3	<3	Mo	<1	<1	V	0.1	<0.1
Cd	<0.1	0.3	Nb	<0.05	0.09	W	<0.2	<0.2
Ce	13.86	0.16	Nd	5.827	0.042	Y	3.44	0.15
Co	<0.05	0.21	Ni	<3	<3	Yb	0.23	0.03
Cr	5	<5	Pb	3.8	0.3	Zn	<5	<5
Cs	0.06	0.18	Pr	1.57	0.03	Zr	<0.1	0.1
Cu	<2	<2	Rb	3.96	5.70			
Dy	0.62	0.01	Sb	0.05	<0.01			

considering, for each element, always the highest value between Test 1 and 2, the bioaccessibility provided by 1 g of FA-Na is from three (Pb) to six (Cr and V) orders of magnitude lower than the limit established by USP<232>. For some elements (e.g. Pb), this is even a conservative appraisal, because bioaccessibility values closer to reality should be those simulating the transit in the whole GI-tract [58]. In fact, the primary mechanism of absorption for most metals is via passage of dissolved species across the small-intestinal epithelium [60], in particular in the ileum, where food remains for the maximum duration [62].

FA-Na complies the acceptance criteria for microbiological quality of non-sterile substances for pharmaceutical use dosage forms, with TAMC and TYMC<10/g, hence below the limits (respectively, $\leq 10^3$ and $\leq 10^2$/g) established for 'non-aqueous preparations for oral use' [35]. Moreover, no evidence of microbial growth was found when the test of sterility was performed on FA-Na heated at 160 °C, a standard procedure to sterilize clays [4]. The X-ray patterns of the material before and after heating are superimposable (Fig. 2), confirming the thermal resistance of this clinoptilolite in Na-form [66].

The textural parameters of FA-Na, determined by gas adsorption, are indicated in Table 6. The specific surface area of FA-Na is 25.2 m^2/g, an intermediate value with respect to those measured on other clinoptilolite-rich powders [55,67]. These parameters are important and have been considered in several studies concerning biomedical applications of zeolites, as they can affect processes such as drug loading and drug release [7,11,31].

Zeta potential measurements performed on FA-Na, showed that clinoptilolite particles have always a negative surface charge whose intensity decreases with the acidity of the solution (Table 7). At pH ≈ 1 (typical of the stomach), the zeta potential is about −10 mV. The zeta potential could be relevant, for example, to assess possible interactions between the material and some drugs with polarity. The point of zero charge (pH_{pzc}) determined for FA-Na is 6.98, as observable in Fig. 3. The value of pH_{pzc} is similar for all three electrolyte concentrations, indicating that the pH_{pzc} is independent of the ionic strength of KNO_3. The curve in Fig. 3 represents the average of the three curves at different concentrations of electrolyte. The plateau from ~5.0 to ~9.5 corresponds to the pH range where the buffering effect of the zeolite takes place. These results confirm that in strongly acidic media the material is not able to vary the pH, as observed in the simulated gastric environment (Test 1), where the final pH was the same of the initial one. Conversely, the pH of a given suspension of FA-Na measured according to USP [38] evidenced a pH variation from 6.85 ± 0.01 to 8.53 ± 0.04.

FA-Na shows a unimodal size distribution curve, reported in Fig. 4. The particle ranges from 0.4 to 80 μm, with a SPAN Index of 1.99. Volume-surface diameter (d_{vs}) and modal diameter (d_m) are 5.32 ± 0.08 μm and 10.83 ± 0.35 μm, respectively. SEM observations of the bulk sample allowed to note that single crystals of clinoptilolite are <10 μm (Fig. 5), but euhedral morphologies were not preserved in FA-Na due to mechanical stress of the beneficiation process. The particle size might be important, because in experiments performed on mice, Martin-Kleiner et al. [20] correlate the intensity of intestinal irritation and inflammation to the different dimension of the administered clinoptilolite-rich powder. This underlines the importance of preparing ingestible powders by maximizing the clinoptilolite content, a mineral with a low hardness (3.5–4), platy shape and an easy cleavage (Fig. 5b), and minimizing the percentage of the commonly associated feldspars and silica polymorphs, harder (6–7) and usually coarser than clinoptilolite. As concerns the grain size, FA-Na complies the requirement established for bentonite by the *Eur Ph.* [52], i.e.,

Table 6
Textural parameters of FA-Na.

S_{BET} (m^2/g)	V_{tot} (cm^3/g)	V_{meso} (cm^3/g)	V_{mic} (cm^3/g)
25.2 ± 0.1	0.132 ± 0.001	0.079 ± 0.001	0.010 ± 0.001

Table 7
Zeta Potential (mV) of FA-Na in water and in HCl solutions at different pH.

Medium	pH	Zeta potential (mV)	e.s.d.
Water	6.05 ± 0.01	−53	±1
HCl 0.0001 M	4.03 ± 0.01	−51	±2
HCl 0.001 M	2.91 ± 0.01	−31	±1
HCl 0.01 M	1.86 ± 0.01	−21	±1
HCl 0.1 M	0.86 ± 0.01	−11	±1

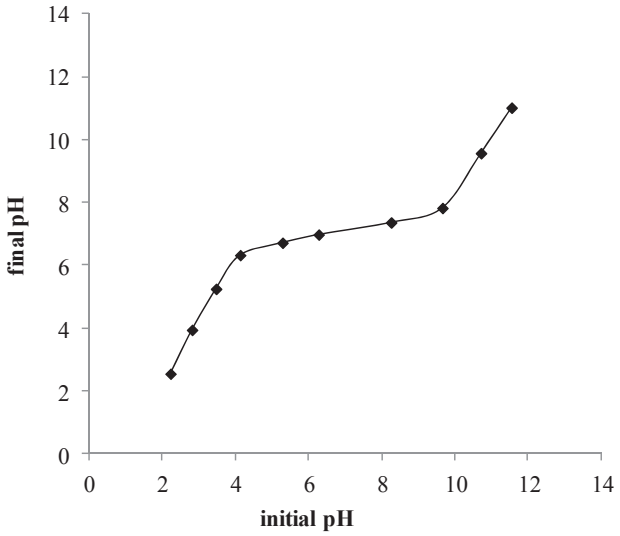

Fig. 3. pH_f vs. pH_i of FA-Na suspension.

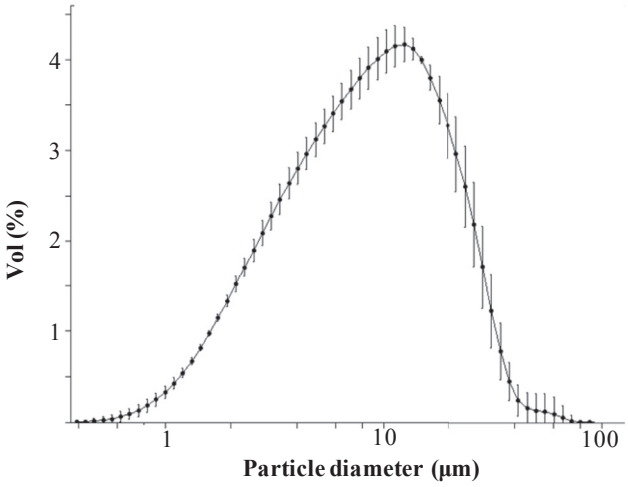

Fig. 4. Size distribution curve of FA-Na particles.

99.5 wt.% of the particles ≤75 μm. Among ingestible clinoptilolite-based products currently marketed, dimensions of about 40 μm are reported [24].

The true density of FA-Na is 2.18 ± 0.01 g/cm^3, a value that matches those measured on pure Na-clinoptilolite crystals

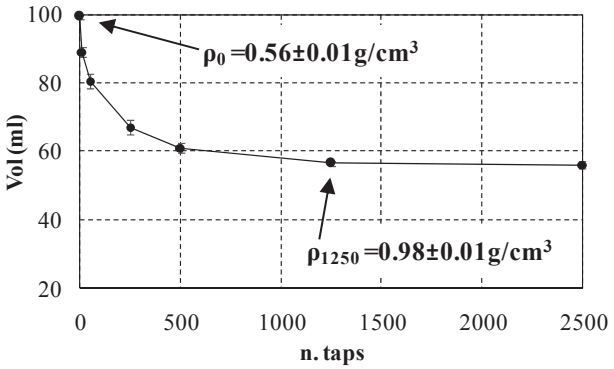

Fig. 6. Volume of FA-Na powders vs. tapping. ρ_0: Bulk density; ρ_{1250}: tapped density.

(2.16 ± 0.02 g/cm^3, [68]), whereas the apparent density of the material increases from 0.56 to 0.98 g/cm^3 after tapping (Fig. 6). The powder shows little inclination to flow, probably because of the hygroscopicity of clinoptilolite, combined with the platy shape (Fig. 5) and the high specific surface, in fact bentonite, that has the same features, is classified as non-flowing excipient [69]. The Hausner Ratio and the Compressibility Index of FA-Na are 1.76 ± 0.01 and 43.00 ± 0.31, respectively, hence between "poor" and "extremely poor" flowability [33]. The water uptake of the material is presented in Fig. 7. FA-Na is able to absorb an amount of water about equal to its weight, with a quick absorption within the first 15–30 s.

4. Final remarks and conclusions

This work reports the preparation and the evaluation of a clinoptilolite-based material, to verify its suitability for pharmaceutical formulations to be administered orally. The mineralogical, chemical, microbiological and technological features of the material were compared, to the largest extent possible, with the recommendations of the most updated rules of International Pharmacopoeias and, in particular, with the specifications for "bentonite", the excipient closest to clinoptilolite already classified by the *Eur Ph.*, USP and JP. The evaluation also considered the data in publications of the International Agency for Research on Cancer, the U.S. Environmental Protection Agency and the World Health Organization Library. It is remarkable that, for the first time, the oral bioaccessibility of potential harmful elements released from a clinoptilolite was evaluated by simulating the transit in the gastrointestinal tract according to the methods recommended by the *Eur. Ph.*

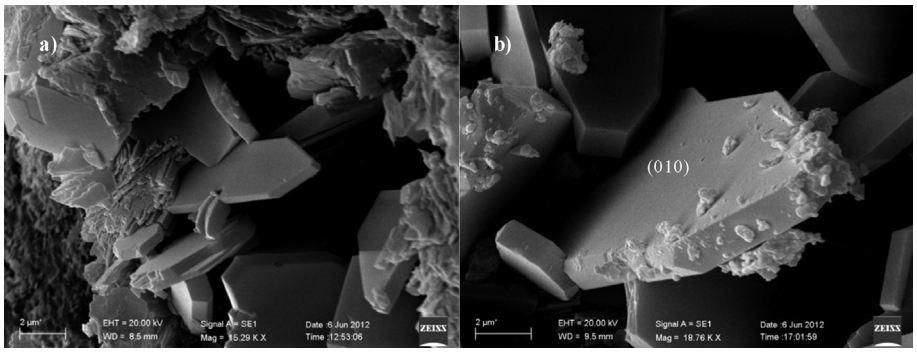

Fig. 5. a–b) Clinoptilolite crystals in the bulk rock; b) zeolites of the clinotilolite–heulandite series show a perfect cleavage parallel to the (010) plane.

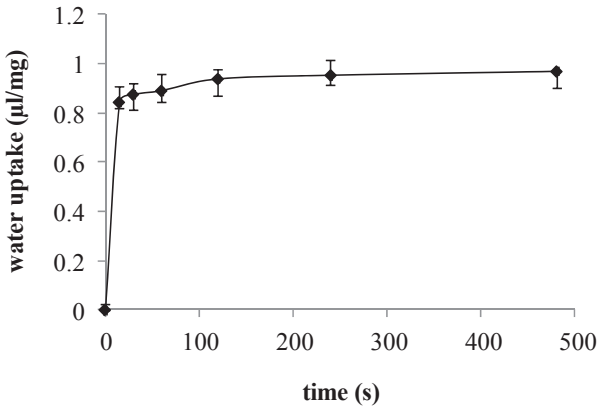

Fig. 7. Water uptake profile.

The prepared material, containing 90.2 wt.% of Na-clinoptilolite, was obtained through a replicable process, and complies the recommendations contained in all the above mentioned sources. Furthermore, its mineralogical and chemical features are comparable with those of marketed ingestible drugs containing clinoptilolite or montmorillonite. This is not sufficient to establish definitely the safety of the material (indeed the proposal of a new medicinal product, that is not among the aims of this work, requires to follow strictly the procedures for the pre-clinical evaluation), but demonstrates the possibility to obtain, starting from a clinoptilolite-rich rock, a reproducible material that maximizes zeolite properties and minimizes any kind of interference chemical, mineralogical and microbiological.

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