G-PUR®

PURIFIED CLINOPTILOLITE

WHITEPAPER

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G-SCIENCE®

G-PUR®/Purified Clinoptilolite Whitepaper

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1 Introductory information

1.1 Disclaimer

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1.2 Contact

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2 G-PUR® – Product Identity

2.1 G-PUR® Composition

G-PUR® consists of purified clinoptilolite. Clinoptilolite is a natural zeolite that occurs mainly in diagenetic products of sedimentary and/or volcanic rocks. The colour of clinoptilolite varies from pale green, blue-green, grey-green, to beige. The solid rock breaks irregularly into sharp-edged, splinter-shaped or board-shaped fragments, with conchoidal smooth to uneven rough fracture surfaces.

2.2 Names and Other Identities

The major raw material of G-PUR® is the mineral clinoptilolite. Clinoptilolite is a crystalline, hydrated calcium potassium sodium magnesium alumosilicate belonging to the group of natural zeolites. In contrast to natural zeolites that are in great quantities found in zeolite-tuff deposits, synthetic zeolites are man-made. The Chemical Abstracts Service (CAS) registry number for clinoptilolite is CAS 12173-10-3.

G-PUR® consists of a clinoptilolite that has been purified by a patented process to minimize the content of contamination by unwanted substances that naturally occur in clinoptilolite.

2.3 Formation of the Raw Material Clinoptilolite used for G-PUR®

In the lower to middle Badenian (approx. 15 million years ago) intensive volcanism led to the emission of large amounts of acidic volcanic ash, which was deposited in the nearby border areas of the Paratethys and subsequently compacted under the growing compressive load of younger volcanic and also clastic sediments. Highly alkaline sea water of the Paratethys was thereby partly trapped in the pore space of the rock. The growing compressive load due to tectonically induced subsidence of the original marine sedimentation space led to pressure and temperature increases in the sediment layers. This triggered the chemical reactions of the alkaline pore water with the acidic rock. Over the course of the following millions of years, the rock went through a weak metamorphosis. During this time the acidic ash material – but mainly the contained volcanic glass components – was partially dissolved under the influence of alkaline pore waters, pressure and temperature. From the resulting (with different elements) highly saturated solution secondary minerals precipitated over time. The main representative of these metamorphosis products is the mineral clinoptilolite [Tschegg et al., 2019; Coombs et al., 1998; Ames, 1960; Sheppard, 1971; Mumpton and Fishman, 1977].

2.4 Clinoptilolite Source/ G-PUR® Raw Material Source

There are many clinoptilolite deposits of differing qualities found around the world, including the

USA, Cuba, Mexico, Turkey, Greece, Ukraine, and China [Margeta et al., 2013].

The presence of impurities and coexisting phases may significantly affect the range of use of clinoptilolites; even within a single mineral deposit, raw material may have varying composition and chemistry. This is the reason why any application of clinoptilolite must be subjected to standardization concerning quality level and processing techniques [Colella, 2011].

G-PUR® is exclusively sourced from a quarry in eastern Slovakia. This huge and very homogenous deposit provides solely clinoptilolite as the predominant mineral phase. Unlike many other deposits, clinoptilolite in this raw material has an extremely low heavy metal content [Tschegg et al., 2019].

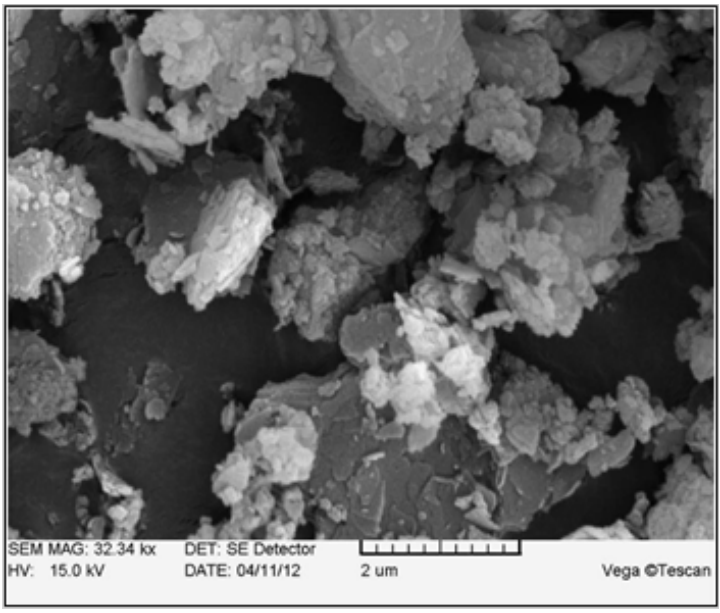

Figure 1 *Picture of G-PUR® taken at high resolution (SE image, magnification 32340, Tescan Vega)*

The fine-grained to aphanitic rock (individual mineral grains are not visible with the naked eye) consists essentially of an extremely fine matrix, in which crystal clasts are embedded – a typical appearance of idiomorphic clinoptilolite crystals can been seen in Figs. 1, 2 and 3. What is unique about the raw material from the Slovakian quarry, apart from its size and homogeneity is the unsurpassed quality of the raw material in terms of high clinoptilolite content, high cation-exchange capacity and low heavy-metal content. No other zeolite phases were identified in the mine [Tschegg et al., 2019].

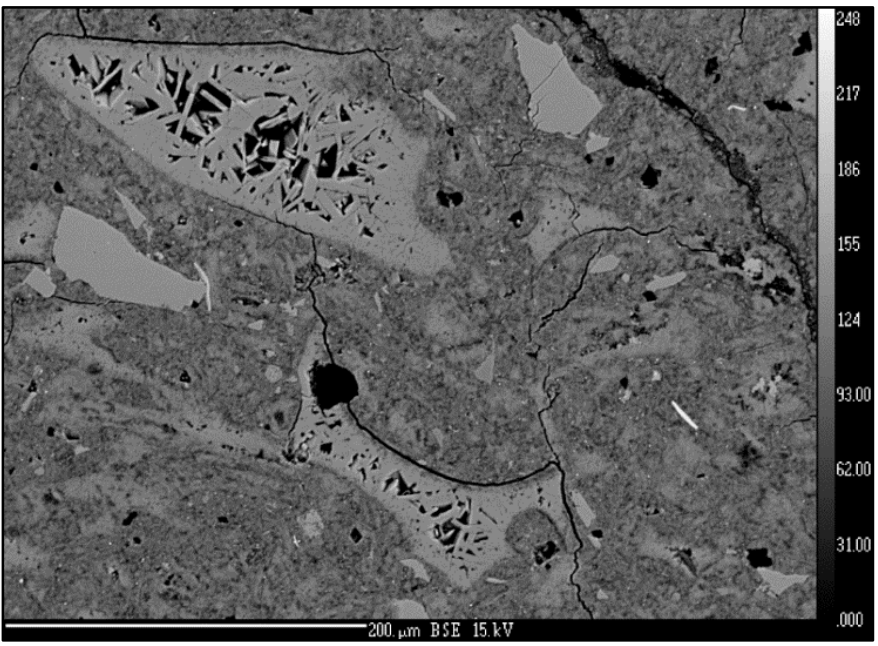

Figure 2 *Electron probe micro-analysis with back-scattered electron imaging of G-PUR® raw material*

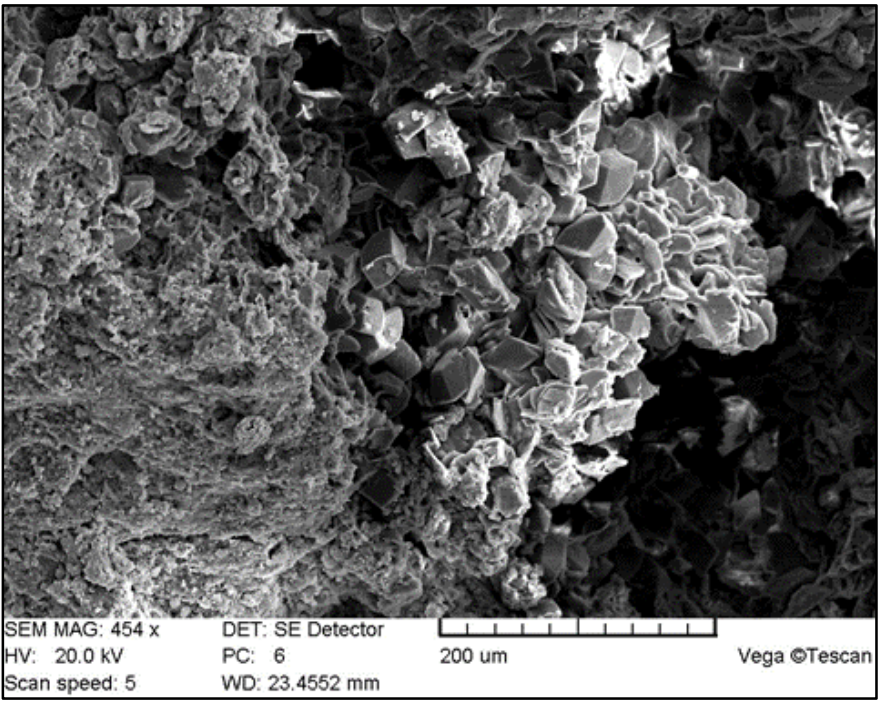

Figure 3 *A section of G-PUR® raw material at low resolution (SE image, magnification 454, Tescan Vega)*

None of the samples from the Slovakian quarry, analyzed by electron microscopy, revealed fibrous or needle-shaped structures. The several hundred quality control samples analyzed by X-ray diffraction for their mineralogical composition did not show traces of such fibrous zeolites (like mordenite or erionite which are both mineralogical similar, but unhealthy zeolites [Stephenson et al., 1999; Baris and Grandjean, 2006]).

2.5 Basic Structure and Characterization

Clinoptilolite is a natural zeolite comprising a microporous arrangement of silica and alumina tetrahedra. The name is derived from the Greek words *klino* (oblique), *ptylon* (feather) and *lithos* (stone). The primary building unit of the clinoptilolite framework is the tetrahedron with 1 silicon or aluminum atom in the centre and 4 oxygen atoms at the verticals. Some of the Si^{4+} is substituted by Al^{3+}. The typical Si/Al ratio for clinoptilolite is > 4. The combination of several primary building units form a three-dimensional structure, the reason why clinoptilolite is part of the tectosilicate group. Channels within the three-dimensional clinoptilolite framework consist of different types: one type consists of the 10-member ring (4.4 – 7.2 Å) and the second type consists of an 8-member ring with a size of 4.1 – 4.7 Å. Besides these two kinds of pore-spaces, clinoptilolite has also meso- and macro-pores with diameters from 10 nm – l10 µm. Macro-pores allow small molecules to reach and adsorb onto the "inner" surface.

Substitution of Si^{4+} by Al^{3+} defines the overall negative net charge and makes clinoptilolite a so called ion-exchanger for positively charged substances (= cations). The negative charge of the framework is compensated by monovalent or divalent cations (called exchange or extra-framework cations) located together with water molecules in structural channels. Cations, which are small enough for exchange mechanism (= smaller than the clinoptilolite pores) can enter or leave the framework. For each ion that is removed from a solution, an equivalent amount of ions from the clinoptilolite channels are exchanged into the solution. The overall structure of clinoptilolite remains geometrically unchanged during this fully reversible process. The silicon and aluminum atoms, as being part of the tectosilicate structure (tetrahedral or framework cations), are not exchanged under ordinary conditions.

The Si/Al substitution leads to possible external surface interactions (sorption effects) either with polar molecules or with other cations, provided that they are large enough to prevent entry into the clinoptilolite channels/pores. These adsorptive effects allow formation of aggregates, characterized in some cases by very high stability (e.g. for cholesterol or microbial toxins like aflatoxins and endotoxins or even some pesticides). The surface area of G-PUR® for such adsorption effects exceeds several hundred square feet per gram [Breck, 1974; Bailey et al., 1999; Baerlocher et al., 2007; Colella, 2011; Spyrnskyy et al., 2008; Petrakakis et al., 2007; Bočarov-Stančić et al., 2011; Armbruster, 2001].

2.6 Human Application – Summary of G-PUR® Characteristics

During digestion, G-PUR® stays inside the digestive tract and is eliminated via the stool. As G-PUR® is stable even in an acid pH environment,

there is no major degradation of the structure. The main effects are ion exchange and surface adsorption. When G-PUR® is in contact with an electrolyte solution, specific extra-framework cations may be removed from their sites within the clinoptilolite and replaced by other cations from the solution, depending on concentration, size and affinity. Due to size, larger cations (like most organic molecules) cannot enter pores (pore size: 4 Å, e.g. sugar: > 8 Å). Despite its large internal surface, G-PUR® has a medium-sized (a few hundred square feet/gram), negatively charged, external surface. This results in possible external interactions either with polar molecules or with other cations, provided that they both are large enough to prevent entry into the G-PUR® channels/pores. Binding capacity to aflatoxins (fungal toxins which can be highly toxic and carcinogenic when consumed in large quantities) is the main reason why clinoptilolite has been used in animal breeding for more than a decade. In Europe, clinoptilolite is mixed with feed and reduces bioavailability of fungal toxins* [Colella, 2011; Ceyhan et al., 2007; Rivera et al., 1998; Pond et al., 1989; EFSA, 2013; Bočarov-Stančić et al., 2011; Dakovic et al., 2000; Katsoulos et al., 2016; Lemke et al., 2001; Vekiru et al., 2015; Li et al., 2008; Armbruster, 2001].

2.7 Serving Form

G-PUR®, in powdered form, is administered in pre-measured 2 gram stick-pack, sachets or 100 gram jars, but capsules or tablets may also be provided.

2.8 Serving Directions

Once daily, prior to a meal, pour 2 g into a glass of water (12 fl oz), stir and drink immediately. Do not take any medication or other supplements within 1 hour of drinking G-PUR®. After 28 days of continual use, discontinue use for 2 days. Do not exceed recommended daily serving. Storage conditions: Store at room temperature; no direct sunlight.

2.9 Excluded Populations

Refrain from use in children. If you are pregnant, nursing, have a medical condition, are using medication, have an intolerance to silicon or aluminum compounds, have limited kidney function or have known chronic gastrointestinal disease, consult a healthcare professional before using.

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2.10 GRAS of alumosilicates

Comparisons to silicate substances generally recognized as safe (GRAS) defined by the FDA are reasonable with the sodium alumosilicate (CFR: 182.2727, as well known as Zeolite A [HERA, 2004; Common Chemistry, 2012]) and the hydrated sodium calcium alumosilicate (CFRcoombs: 182.2729; a subtype of zeolite A) as these minerals – beside a comparable composition – hold a similar crystal structure. Both analogues however are synthetic and have differing oxide ratios. The sodium aluminum silicates have Na_2O:Al_2O_3:SiO_2 oxide ratios of approx. 1:1:3, whereas clinoptilolite has approx. 1:10:64. Considering the remaining alkaline and earth-alkaline elements that compose the clinoptilolite (not solely Na_2O) results in a (K_2O+Na_2O+CaO):Al_2O_3:SiO_2 ratio of 1:1.5:9. The structural relationships are given by cage-like structures of both the synthetic alumosilicates and the G-SCIENCE®, Inc. purified clinoptilolite, forming porous crystals, which allow an uptake and exchange of 'guest' ions. A comparison to the other GRAS silicates cannot be drawn due to different chemical (no or insufficient aluminum like for example calciumsilicate, CAS 1344-95-2) and structural properties [Baerlocher et al., 2007; Lobo 2003].

The main difference between the synthetic GRAS silicates and G-SCIENCE®, Inc. purified clinoptilolite is that clinoptilolite is of natural origin, contains not only sodium but also potassium, calcium and magnesium, and the stability in acid environment is very high, compared to synthetic analogues.

3 Supportive Information

3.1 Chemistry/Identity

3.1.1 Physical Properties of Clinoptilolite

The physical properties for clinoptilolite from the Slovakian quarry are summarized in Table 1.

Items	Description
CAS No.	12173-10-3
Color/Form	grey-green
Odor	none
Physical state at 20°C	solid
Solubility in water	none
Melting point	1340°C
Flow temperature	1420°C
Ignition temperature	settled dust – neg. up to 600°C raised dust – neg. up to 800°C
Flash point	up to 600°C neg.
Explosive limits (volume % in air)	non-explosive
Specific gravity	2200 - 2440 kg/m^3
Density	1600 - 1800 kg/m^3
Porosity	24 - 32%
Pore diameter	0.4 nm (4 Å)
Mohs hardness	1.5 - 2.5
Specific surface (BET)	30 - 60 m^2/g
Thermal stability	up to 400°C

Table 1 Physical properties of clinoptilolite from the Slovakian quarry [IZA 2010; Vatalová et al., 2016]

3.1.2 Dissolution/pH Stability

Clinoptilolite is not soluble in water and is highly resistant to acid environments, which is very important if applied to the human body. A comprehensive study of the effect of pH on the chemical stability of clinoptilolite was conducted by Li et al. [2008]. The authors exposed clinoptilolite samples to aqueous solutions of nitric acid at a pH range between 1 to 5 for durations of 2, 12, 24, 48, 72, or 144 hours. The pH was maintained constant throughout the duration of the experiment. At each time-point, samples of the supernatant were measured for concentrations of the major elements Al^{3+}, Si^{4+}, Na^{+}, and K^{+} for evidence of acid leaching using inductively coupled plasma mass-spectrometry (ICP-MS). Samples treated at each pH value for 144 hours were also evaluated for evidence of qualitative changes in chemical structure or mineralogical morphology using XRD, energy dispersive X-ray fluorescence (ED-XRF), and transmission electron microscopy (TEM). The authors described that no significant quantities of Al^{3+}, Si^{4+}, Na^{+}, and K^{+} were leached out of the material at pH values of ≥ 2. X-ray diffraction patterns demonstrated that the crystallinity of clinoptilolite is highly resistant to pH as no effects on the major peaks within the X-ray diffraction pattern were observed even at a pH of 1 over 144 hours. Furthermore, the high stability of the material was reflected in the absence of significant changes in TEM (transmission electron microscopy) morphology at pH ≥ 2. Findings from this study demonstrate that dissolution of G-PUR®, during gastric transit, is not expected [Li et al., 2008].

3.1.3 G-PUR® Raw Material

The G-PUR® raw material is obtained exclusively from the Nižný Hrabovec volcanic tuff deposit in eastern Slovakia. The deposit is characterized by the stable occurrence of clinoptilolite (mostly K and Ca, with the formula $(Ca_{1.51}K_{1.39}Mg_{0.37}Na_{0.15})[Al_{5.64}Si_{26.36}O_{72}]*11.77\ H_2O$ [Tschegg et al., 2020]) of extremely high purity and homogeneity (for further detailed information on the quarry as well as the raw material please see Tschegg et al. [2019 and 2020]). The raw material, before being used for manufacturing G-PUR®, is inspected optically for Mn/Fe-oxide staining and tested by elemental analysis (ICP-MS) for inorganic impurities; the qualitative identity of the raw material is assured by X-ray diffraction (XRD) and analysis of the ion-exchange capacity (IEC). A typical chemical composition is summarized in Table 2.

Parameter	%
SiO_2	65 - 75
Al_2O_3	9.5 - 14
CaO	2.0 - 5.6
MgO	< 1.3
Na_2O	0.2 - 2.0
K_2O	1.5 - 4.5

Table 2 Typical major elemental composition of G-PUR® raw material

3.1.4 Quality Control/Identity Verification of G-PUR®

It is important to emphasize that a complete characterization of any clinoptilolite before use in the biomedical field is a prerequisite. Clinoptilolite raw materials may be "different" depending on where they are mined and they cannot be proposed for any use as being identical. Moreover, standardization is necessary and suitable criteria must be defined and followed for each specific application. These must include the mineral composition and absence of undesired minerals, the exact particle size distribution and milling technique, the elemental composition with emphasis on heavy metal content and the functionality by measuring the ion exchange capacity [Colella, 2011].

The quality and identity of G-PUR® is established by the following specific analytical characteristics:

- Determination of the elemental composition.
  Method: ICP-MS after pressure acid digestion.
- Determination of the crystalline constituents.
  Method: X-ray diffraction.
- Determination of the particle size distribution.
  Method: laser diffraction.
- Determination of the specific ion exchange capacity.
  Method: ion chromatography.

Identification via NMR, UV or mass spectrum is not possible and/or conclusive due to the specific properties of clinoptilolite.

3.2 Manufacturing Process

The purification process comprises a thoroughly validated and quality-controlled manufacturing procedure, from the incoming goods inspection of the raw-material to the release of the final product. The used production equipment is qualified to fulfil FDA standards; process and facility are under control of strict hygiene measures. G-SCIENCE®, Inc. is a FDA-registered food facility as is the manufacturer Glock Health Science & Research GmbH.

All incoming lots of raw material are inspected to meet the strict specifications. The raw material is then processed with the proprietary, patented purification process to minimize the – naturally occurring – impurities of the raw material. During manufacturing, numerous quality control steps guarantee a constant, homogeneous and reproducible quality of the final product. The processing results in the desired depletion of the unwanted impurities and the process parameters guarantee that no cations of the materials are bioavailable any more. No mineralogical differences regarding the crystalline structure are observed between the un-processed raw material and the purified material (G-PUR®). Similarly, the purification process alters the ion exchange capacity of the material, a qualitative and very important characteristic. At the end of the production process, G-PUR® is milled to a mean particle size of 3.1 µm (d50). The special milling technique, unlike techniques that use a grinding media like a ball mill, guarantees minimal damage to the crystal structure of G-PUR®. Additionally, it adds almost no contamination to the milled product, as there is no wear of grinding balls etc. All necessary energy is delivered by clean compressed air, the particles are micronized by particle-particle collisions. The mean particle size of 3 microns ensures a maximum efficacy concerning the active surface of G-PUR®. Finally, G-PUR® is processed to an ultimate heating step (equal to dry sterilisation). The bulk is filled into food grade plastic bins, sealed, shipped to the US and packaged by a certified contract manufacturer.

3.2.1 Process Controls

Manufacturing of G-PUR® is performed according to processes defined in so called standard operating procedures. All stages of production as well as all media that get in contact with G-PUR® during production are of certified, high-grade quality. The production process is closely monitored by a certified quality-management system and a highly sophisticated lab with validated, state-of-the-art chemical, microbiological, and mineralogical analysis devices and methods to verify compliance for all relevant quality parameters of the final product.

3.2.2 Quality Management

Documentation of the entire production is supervised and controlled by an EN-ISO 13485 (for medical devices) certified quality management system. During the production process, more than 30 checklists are filled out – consistently documenting the whole production process, from the incoming goods inspection to the shipping. Traceability down to the exact position of the origin of the raw material in the quarry can be guaranteed for every batch of G-PUR®. Production equipment has been fully qualified with a group of external specialists to fulfil all regulatory requirements that apply.

3.2.3 Shelf Life

G-PUR® consists of a mineral that is absolutely stable against normal degradation. To guarantee microbiological stability, the humidity is strictly controlled as part of the product specifications as well as testing the absence of E.coli and limiting the maximum cfu (colony forming unit) per dose. Microbiological control is not only restricted to the product itself, but also performed for the surrounding environment (air, surface etc.) and the media (compressed air, water) which get in contact with G-PUR®. Furthermore, G-PUR® offers little to no nutritional value for growth of microorganisms. A stability study has been performed and resulted in a minimum shelf life of 3 years.

4 Intended use – Health Benefits*

4.1 Binding of Dietary Cholesterol

4.1.1 Preface

According to published scientific literature, zeolites do adsorb sterols. Zeolites have a particular affinity for molecules with a permanent dipole, and sterols have a dipole due to the -OH group in the ring system. Ground clinoptilolite bears a charged surface of several hundred square feet per gram, and polar molecules that are big enough to prevent them from entering the pores can be adsorbed onto this surface. The complex with cholesterol shows a very high stability, as described by Berezin et al. [2001]. In an artificial matrix, adsorption of cholesterol by zeolite appeared to be irreversible. Adsorption proceeded until there was either no sterol left in solution or the zeolite was saturated. Furthermore, treating the saturated zeolite with fresh hexane removed no sterol; hot ethanol had to be used for desorption. Though the zeolite described by Berezin et al. [2001] wasn't clinoptilolite but a synthetic zeolite, structural similarities justify the extrapolation to clinoptilolite* [Berezin et al., 2001; Colella, 2011; Mumpton, 1985].

4.1.2 Animal data

In a study with rabbits, a significant interaction could be detected between dietary cholesterol and G-PUR® *. 12 New Zealand white rabbits were divided into 4 groups (n = 3) and were fed according to the following scheme:

Group	Days 1 - 7	Days 8 - 21
1	Normal diet	Normal diet
2	Cholesterol diet + 1.5% Chol	Normal diet
3	Cholesterol diet + 1.5% Chol	Cholesterol diet + 1.5% Chol
4	Cholesterol diet + 1.5% Chol	Cholesterol diet + 1.5% Chol + 10% Clinoptilolite

Table 3 Study group assignment

The control-diet contained very little cholesterol (0.4 g per kg feed based on dry weight); whereas the cholesterol diet was made up of a special feed with a comparable concentration of dietary fibres (13.3 % compared to 14.9 % in the control diet), the addition of 7 % peanut oil, and 1.5 % cholesterol. Study group 1 received the normal feed (control diet) during the whole test period. Group 2 received the cholesterol diet on days 1 to 7, then on days 8-21, group 2 was switched back to the normal diet (control diet). Groups 3 and 4 received the cholesterol diet throughout the entire study period, with group 4 also receiving 10 % clinoptilolite (by weight) as a supplement to the cholesterol diet from days 8-21. Researchers

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collected blood samples from the animals on days 1, 5, 8, 10, 12, and 19.

After an administration period of 21 days, the researchers conducted histological examinations of the animals, which included the assessment of the lungs, heart, small intestine (incl. Peyer patches), liver, spleen, and kidneys. In addition, a collective faecal sample was taken for a period of several days and, *inter alia*, was analysed for the cholesterol content. The results of the serum analysis on day 21 of the study are shown in table 4:

Based on the comparison of group 4 with the group 1 control and group 2, which switched back to a normal diet on day 8, it is obvious that the addition of clinoptilolite to the feed reduces the bioavailability of dietary cholesterol and/or the dietary cholesterol resorption significantly and subsequently influences the biochemical markers of cholesterol supplementation*. LDL, HDL and triglycerides did not increase as much in group 4 due to the supplementation of clinoptilolite compared to the high cholesterol diet of group 3 without the clinoptilolite supplementation*.

Parameter	Normal Diet	Cholest. Diet + Normal Diet from Day 8	Cholest. Diet + Clinopt. from Day 8	Cholesterol Diet
HDL (mg/dl)	44.5	175.6	126.0	562.0
SD (mg/dl)	4.6	48.6	32.2	241.5
LDL (mg/dl)	24.5	650.9	538.1	1708.2
SD (mg/dl)	5.6	153.0	90.9	656.4
Trigly (mg/dl)	47.8	88.8	106.2	224.1
SD (mg/dl)	3.8	33.7	52.3	92.6
Cholest (mg/dl)	67.3	753.3	622.2	2335.0
SD (mg/dl)	1.4	168.2	96.5	929.6

Table 4 *Cholesterol/LDL/HDL and triglyceride serum concentrations of all study groups on day 21 (mean and standard deviation SD)*

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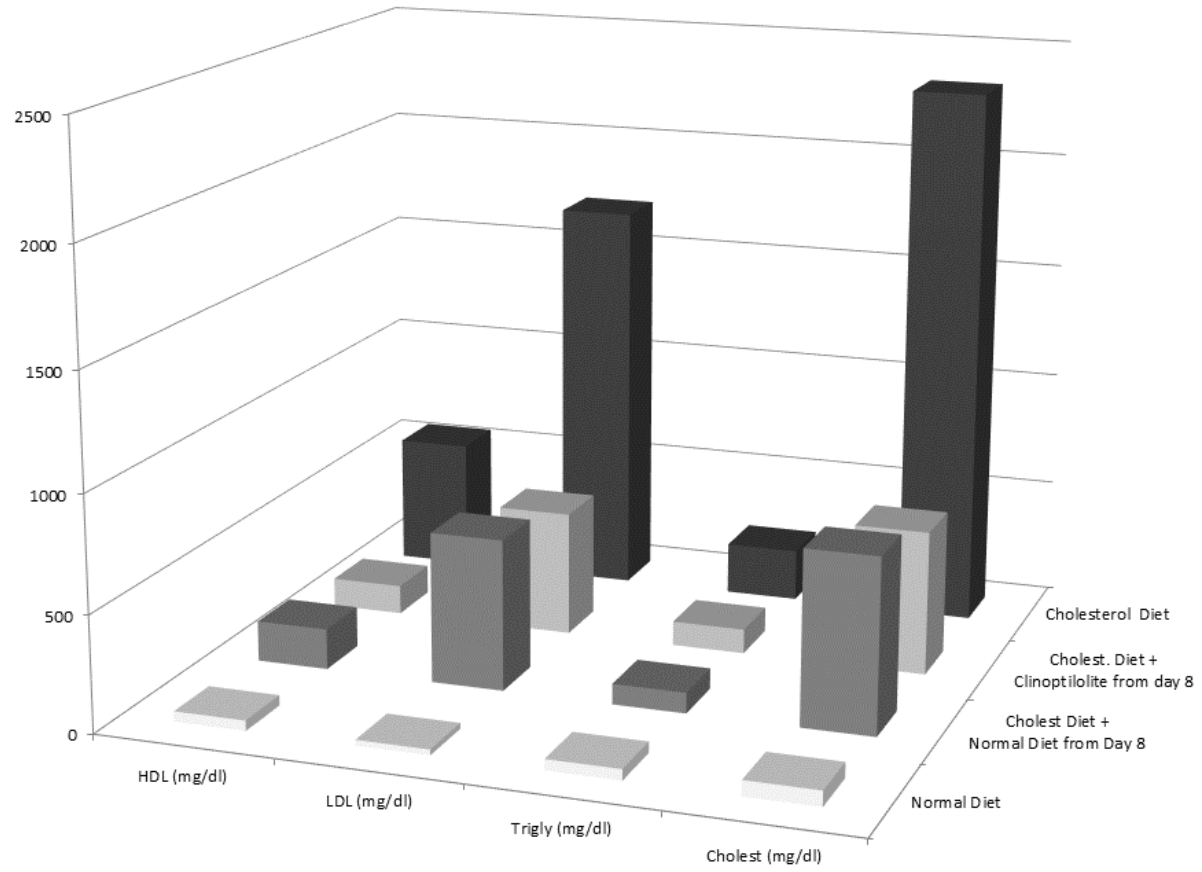

Figure 4 *Cholesterol/LDL/HDL and triglyceride serum concentrations of all study groups on day 21*

The illustrated cholesterol/LDL/HDL and/or triglyceride concentrations of group 2, which received normal feed after day 8, and group 4, which received the feed fortified with cholesterol plus the 10 % clinoptilolite supplementation, cannot be differentiated from each other with statistical significance ($p > 0.05$). With the addition of clinoptilolite, the serum cholesterol markers did not appear to rise despite the increased cholesterol consumption, suggesting the clinoptilolite was not allowing the dietary cholesterol to be resorbed by the body. In other words, continuing with a high cholesterol diet with the addition of clinoptilolite resulted in the reduced bioavailability of cholesterol and subsequently no further increase of the relevant serum parameters* [Losert, 2004].

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This is further substantiated as the cholesterol analysis from the faeces (data not shown) revealed that the analytical method, which normally involves an extraction step, could not recover all of the extra cholesterol that was applied with the fortified diet. This might be due to the very strong cholesterol-clinoptilolite interaction, which may even resist to desorption with solvents* [Folch et al., 1956; Lee et al., 1994; Berezin et al., 2001].

On the basis of the above results, a reduction of the bioavailability of dietary supplied cholesterol by addition of clinoptilolite to a high cholesterol diet is supported*.

4.1.3 Lab data

The following G-SCIENCE®, Inc. lab data support the idea that clinoptilolite helps bind dietary cholesterol in the digestive tract (even in a high fat matrix) so it cannot be resorbed by the body, as a very strong interaction between cholesterol and clinoptilolite is formed, which is extremely stable.*

Laboratory tests were conducted to study the interaction of clinoptilolite with cholesterol in a vegetable oil matrix doped with cholesterol. Cholesterol content was determined before and/or after incubation of the vegetable oil matrix doped with clinoptilolite.

total cholesterol quantity [mg]	adsorbed by Clinoptilolite [mg]	adsorbed percentage [%]
23.3	11.5	49.3
46.5	19.1	41.1
116.3	30.8	26.5
232.7	39.9	17.2
465.3	69.4	14.9
2326.7	322.9	13.9

Table 5 *In-vitro cholesterol adsorption per 1 g G-PUR®*

The results clearly show that even in a complex, high-fat matrix there is a significant interaction between G-PUR® and cholesterol*.

In order to verify the robustness of the adsorption of cholesterol on clinoptilolite in other complex matrices, experiments were carried out with bovine serum that was brought into contact with clinoptilolite and incubated at 37 °C. Bovine serum contains a high amount of protein, lipids, carbohydrates, amino acids and electrolytes. After subsequent separation of the clinoptilolite, relevant clinical parameters were analyzed from the clinoptilolite/serum samples, as well as from a control without clinoptilolite, in order to examine the specific influence of clinoptilolite in this matrix.

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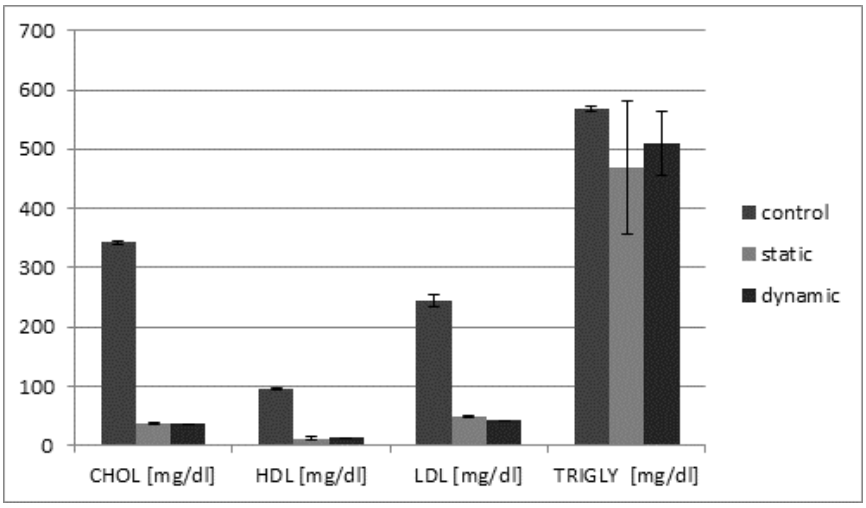

Figure 5 *Lipid concentrations after incubation of bovine serum with G-PUR®*

For the parameters total cholesterol, HDL and LDL, a statistically significant reduction in concentration was found in the serum that had been exposed to clinoptilolite when compared to the control bovine serum sample. This could be observed for a static as well as a dynamic model with regular mixing of the sample by means of a rotator. These results clearly show that specific adsorption of cholesterol by clinoptilolite occurs, even in a highly complex matrix like bovine serum*.

Due to the technical feasibility, both experiments were carried out with significantly bigger particle sizes than those of G-PUR®. Taking into consideration that the effect of G-PUR® to bind cholesterol is mainly a surface effect, it could be expected that this effect is even more pronounced for finer particle sizes (smaller particle size/larger surface area)*.

Berezin et al. [2001] conducted *in vitro* studies to analyze the interaction of zeolites with sterols. An artificial mixture of different sterols was incubated with zeolite and the interaction was examined. In particular for cholesterol, Berezin et al. [2001] was able to prove that the amount of cholesterol in the mixture was reduced after mixing with zeolite. Furthermore, within the closely analogous sterols, the affinity to cholesterol was the greatest. The interaction between the sterols and the adsorbent was so strong that desorption of the adsorbed sterols with fresh hexane was not possible and succeeded only with the addition of hot ethanol. Stability of the sterol after contact with zeolite was verified by subsequent analysis of the sample medium*. [Berezin et al., 2001]

The fact that zeolite can adsorb sterols is due to the affinity of the zeolite for molecules with a

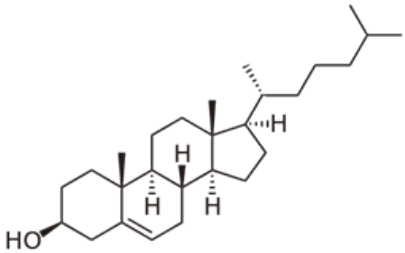

Figure 6 Cholesterol structural formula

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permanent dipole, as is inherent for instance with sterols of the OH group. This can also be extrapolated to the structurally similar clinoptilolite, which also has a charged surface and a high affinity to adsorb substances with a dipole* [Mumpton, 1985; Sprynskyy et al., 2008; Colella 2011; Berezin et al., 2001].

Summarized, the totality of *in-vivo* and *in-vitro* data undoubtedly suggests that addition of G-PUR® to diet might significantly reduce bioavailability of dietary cholesterol in the digestive tract*.

4.2 Binding of Heavy Metals*

4.2.1 Animal Data

Pond et al. [1989] evaluated the effect of clinoptilolite and a synthetic zeolite (NaA) against cadmium-induced anaemia in swine. For 31 days, swine aged 4-5 weeks were administered a diet that contained 94 ppm Cd (WHO guideline for drinking water is 3 ppb) as $CdCl_2$. The clinoptilolite administered at a dose of 3 % (w/w) to feed originated from Castle Creek, Idaho, and consisted of 66 % clinoptilolite. Particle size was smaller than 50 mesh or < 300 microns. Liver Cd concentration was increased dramatically ($p < 0.01$) by dietary Cd, while haematocrit and haemoglobin were significantly reduced after 28 days of Cd administration, showing that the dietary Cd was highly bioavailable. Livers of pigs fed clinoptilolite and Cd had significantly lower Cd concentrations than those of pigs fed Cd alone ($p < 0.01$)*.

There was also a tendency for clinoptilolite to lower the concentration of Cd in the kidney, but this effect failed to reach statistical significance. Another observation made indicated the Fe content did not decrease in swine consuming clinoptilolite with the Cd diet. This supports the theory that some of the dietary Cd may has been absorbed to the clinoptilolite matrix in accordance with the known ion-binding capacity. Its protection from Cd resorption implies maintenance of appreciable integrity of the crystal structure and Cd binding capacity into the duodenum where most Fe resorption occurs. The failure of clinoptilolite to affect plasma concentrations of urea-N, K, Na or Mg suggests no major shift in plasma electrolyte compounds due to the administration of clinoptilolite* [Pond et al., 1989].

The fact that in this study clinoptilolite administration has not been able to significantly lower the Cd accumulation in the kidney may be due to the very high concentration of Cd in the feed. Besides that, clinoptilolite clearly prevented dietary Cd from being resorbed in a pig animal model*.

The binding of heavy metals with the subsequent reduction of their bioavailability is described in

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two publications [Beltcheva et al., 2012; Topashka-Ancheva et al., 2012]. Reduction of bioavailability was examined after an administration period of 90 days via the lead concentration of different organs of mice and concurrent administration of drinking water with 0.005 M of lead nitrate solution with the addition of clinoptilolite in feed. The lead concentration in the examined organs (carcass, liver, kidneys, and bones) and also the feces could be reduced by 77 % to 90 % when clinoptilolite was added, implying that clinoptilolite reduced the bioavailability of the dietary lead. Additionally, results of the feces analysis suggest that a large amount of the Pb administered was bound to the clinoptilolite in feed and could not be recovered with the analytical method used. As the digestion mixture for analyzing organs consisted of nitric and perchloric acid without the addition of hydrofluoric acid, it seems quite reasonable that feces samples with a high content of inorganic clinoptilolite could only be partially extracted instead of completely dissolved, resulting in much lower recovery rates. This further substantiates the theory that interaction between heavy metals and clinoptilolite can be very strong, significantly reducing the bioavailability in the digestive tract* [Beltcheva et al., 2012; Topashka-Ancheva et al., 2012].

In another study, ICR mice were held under identical conditions and administered lead in their diet, with some groups also receiving supplementary clinoptilolite. After 90 days of treatment, a 2.3-fold reduction of the occurrence of chromosomal aberrations was observed in the mice that consumed a diet fortified with lead and received clinoptilolite supplementation when compared to mice that consumed the lead-fortified diet but did not receive clinoptilolite; one by a 2.5-fold increase of the mitotic index, one by a 1.5 factor increase of normal erythrocytes, and one by a 1.3 factor increase in body weight. On day 90, the mean kidney Pb concentration in group 4 (receiving Pb and clinoptilolite) was 11-fold lower than that in group 3 (receiving only Pb). Respectively, on day 90, the Pb concentration in bones of the mice from group 4 was 4.3-fold lower compared to Pb concentration in bones of mice from group 3, clearly indicating that less dietary Pb was bioavailable in group 4*.

Chromosomal aberrations, involving gross alterations of genetic material, have been considered a sensitive endpoint for detecting genotoxic effects induced by heavy metals and toxic chemicals. Thus, the study of cytogenetical status is considered highly relevant in the human context. In the experiment described above, the increased percentage of aberrant cells in the mice from group 3 is likely due to the very high concentration of bioavailable Pb* [Pottier et al., 2013; Garcia-Leston et al., 2010].

Micronuclei in erythrocytes of peripheral blood were observed in the specimens from groups 3 and 4. In group 4, they appeared sporadically mainly before day 45. This fact indicates that the main part of Pb entering in the mouse organism via drinking water has been captured by the clinoptilolite sorbent, and thus, the Pb quantity resorbed by the intestinal mucosa and utilized by the organism at the cellular level had been significantly reduced*.

In summary, chromosome aberrations, lowered mitotic index, pathologically changed erythrocytes, diminished erythropoiesis, and reduced body weight gain of the Pb-exposed laboratory mice demonstrated a well-expressed toxicological stress due to the chronic exposure to bioavailable Pb. The structure of chromosomes and red blood cells as well as the mitotic index and erythropoiesis was significantly improved by clinoptilolite supplementation. The time course of the body weight in the clinoptilolite and Pb supplemented animals did not differ significantly from that in the control mice. The authors concluded these differences were a result of reduced amounts of Pb entering the blood, organs, and bone marrow of the clinoptilolite-supplemented mice due to the high ion exchange capacity of the administered clinoptilolite – suggesting that bioavailability of Pb has been significantly reduced by the supplementation of clinoptilolite* [Beltcheva et al., 2012; Topashka-Ancheva et al., 2012].

Due to their ion-exchange capacity, Pb bioavailability and Pb resorption could be reduced by adding clinoptilolite to the diet, to maintain a healthy body system* [Papaioannou et al., 2005].

4.2.2 Lab Data

Numerous studies suggest that clinoptilolite has a high affinity for heavy metals, and therefore, can be used to treat different matrices (wastewater, soil, drinking water etc.) to reduce the concentration of bioavailable heavy metals [Shaheen et al., 2012; Sharifipour et al., 2015; Wingenfelder et al., 2005; Erdem et al., 2004; Ghasemi-Fasaei et al., 2012; Kragović et al., 2012; Minceva et al., 2007; Elizalde-González et al., 2001a/b; Faghihian et al., 1999; Vaca Mier et al., 2001; Delkash et al., 2015; Puschenreiter et al., 2005; Chojnacki et al., 2004; Dursun and Pala 2007].

Buasri et al. [2008] investigated the ability of clinoptilolite to remove lead from aqueous solutions. The results clearly showed that adsorption onto clinoptilolite is a very rapid process, even in an acidic pH environment at elevated temperature. At a concentration of 2 g clinoptilolite per 100 ml, even at pH 2 adsorption

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capacities reached > 30 mg lead per g clinoptilolite for a very coarse grain size of 75 – 150 µm. For a concentration of 100 ppm lead, adsorption onto clinoptilolite was complete after 30 minutes contact time. Coarser particle size resulted in a decrease in adsorption capacity. When compared to other adsorbents like clay, organic adsorbents or activated carbon, clinoptilolite adsorption capacity was comparable and within the same order of magnitude* [Buasri, 2008].

Given the fact that G-PUR® has a much finer particle size than the 75 – 150 µm used, effects may be even faster with G-PUR® compared to those observed by Buasri et al. [2008]. Adsorption efficacy at pH 2 is important to further verify that effects are also taking place in an acidic environment*.

In a study of Petrakakis et al. [2007] the leachability of lead (Pb^{2+}) from natural clinoptilolite of Greek origin, batch-loaded with lead, was investigated according to standard procedures. Subsequently, the influence of pH, initial load of lead in clinoptilolite, agitation rate, temperature, and particle size on kinetics of lead leaching was also studied. Fractions 1.4 – 2 and 5 – 6 mm were used in the study. Sorption was fast during the first few hours and equilibrium was practically attained after 24 h. The loaded samples had 12 – 103 mg Pb^{2+}/g clinoptilolite. For leaching experiments, samples used contained typically 30 mg Pb^{2+}/g clinoptilolite. At pH 1 (HCl), more than 18 % of the lead could be leached from the (pre-loaded) clinoptilolite. At higher pH, leaching rates dropped below 1 %, showing high stability of the lead-clinoptilolite complex at pH 2 or higher. Lead solubilization experiments were also performed with water (tap or deionized) at 30 °C for 24 h and in all cases less than 0.02 % of the Pb initially loaded was detected in the solution. Particle size did not appear to significantly alter the leachability of lead from pre-loaded clinoptilolite* [Petrakakis et al., 2007].

These results suggest the lead-clinoptilolite complex can be characterized by a very high stability in an environment at pH > 3. Due to the high pH in the human intestine, release of the absorbed Pb within the intestine seems highly unlikely*.

Batch adsorption kinetic and isotherm studies were conducted by Payne and Abdel-Fattah [2004] to compare and evaluate different types of adsorbents for lead ion removal from aqueous media. The effects on lead ion absorption from pH changes, competing ions, and temperature increases were also investigated. Besides natural clinoptilolite, several other natural and synthetic adsorbents were investigated. Natural clinoptilolite had a surface area of 40 m^2/g and a

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Si:Al ratio of 6:1. The relative rate for lead ion adsorption was: 13X > chabazite > clinoptilolite > 5A > activated carbon. Clinoptilolite Pb removal rate increased from 40 % to > 80 % if pH was raised from pH 2 to > pH 3 and was always > 80 % for pH > 3. Ionic competition reduced lead ion removal by the zeolites: compared to DI water with an adsorption rate of almost 80 %, addition of 0.01 M KNO_3 or 0.1 M KNO_3 reduced effectivity to 50 % and 37 %, respectively. Increasing temperature from 23 °C to 35 °C significantly improved adsorption performance for clinoptilolite* [Payne and Abdel-Fattah, 2004].

Despite the fact that increase in temperature also increases the absorbent performance, it is worth noting that even for a matrix with elevated levels of other cations like potassium, there was still a significant interaction between clinoptilolite and lead, resulting in a reduction of available Pb in solution*.

Tests under standardized lab conditions as well as verification of these results in animal models indicate that G-PUR® might interact with heavy metals, resulting in stable complexes that can significantly reduce bioavailability of dietary heavy metals in the digestive tract*.

4.2.3 Clinical Data*

In the human body, lead is manly found in the teeth and bones, where up to 94 % of the lead total body burden concentrates. The turnover of the latter determines the long half-life of 20-30 years. The tight binding of lead to hydroxyapatite results in continuous accumulation in the human body [Barry, 1975]. The fraction of lead circulating in the blood, shows a half-life of approximately 28-40 days before being eliminated by excretion in urine and faeces [Rabinowitz et al., 1976]. Not only long time but also short exposure to lead can have a substantial effect on almost all parts of the organ system.

In a randomized, placebo-controlled, double-blind, parallel-group study, 42 healthy, fasting participants were randomized in 3 groups, receiving orally one dose of: 2 g G-PUR®, 2x 2 g G-PUR®, or placebo, together with 2.5 µg of ^{204}Pb in water. The stable lead isotope ^{204}Pb was used as a tracer and its enrichment in urine and blood was determined by ICP-MS. Concomitant oral intake of G-PUR® reduced the enteral uptake of the tracer lead in healthy humans by approximately 90 %. The mean maximum of ^{204}Pb enrichment of 0,505 % of total blood lead was significantly higher ($p<0.0001$) in the placebo group compared to the groups receiving 2 g G-PUR® (0.073 %) or 2x 2 g G-PUR® (0.057 %). Although the uptake of

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^{204}Pb was not delayed by concomitant purified clinoptilolite tuff ingestion (similar t_{max} values between the groups were described), G-PUR® reduced the bioavailability of the tracer as seen as a decrease in blood and urine. G-PUR® was well tolerated and no relevant nor new risks were identified* [Samekova et al., 2021].

4.3 Reduction of the Bioavailability of Mycotoxins*

Mycotoxins are metabolic byproducts of molds, which – even at a very low dose – can be toxic to invertebrates. Many regulatory authorities throughout the world have established low tolerances for mycotoxin contamination in food and feed in order to provide an adequate protection against the potential negative effects of these toxins in animals and humans. This is highly important as, due to their very high stability, even processing of food (i.e. cooking) does not destroy most of the mycotoxins, so they may accumulate in various sources and find their way into the human diet [Bullerman and Bianchini, 2007; Bosco and Molle, 2012; Marin et al., 2013].

4.3.1 Clinical Data*

An application of clay minerals for detoxification and/or reduction of the bioavailability of aflatoxins (AF) has been shown to be safe in phase 1 and phase 2 human studies. The calcium-montmorillonite used in the study is also an alumosilicate, like clinoptilolite, but it belongs to the group of phyllosilicates. Both substances consist mainly of the elements aluminum and silicon, but are different in their structure. However, concerning their principal sorptive properties and their basic affinity for aflatoxins, the materials are comparable*.

In a 3-month, double-blind and placebo-controlled, phase II clinical trial in adults, dosages of 1.5 g (LD) or 3.0 g (HD) of the clay material per day showed a statistically significant reduction of biomarkers of the aflatoxin burden of the test subjects. AFM1 levels in urine samples were analyzed as well as AFB1 albumin adduct in serum. Levels of urinary AFM1 and serum AFB1 albumin adduct have shown significant correlation with dietary intake of aflatoxins. AFB1 albumin adduct is also the most reliable molecular biomarker for studying human exposures to aflatoxins. About 2 % of the ingested AFB1 is reported to be covalently bound to serum albumin. For both the low and the high dose group, AFB1 albumin adducts in serum significantly decreased after 3 month. This delay is probably attributable to the long half-life of albumin, which is estimated to be approximately 3 weeks in healthy people. However, statistically

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significant decreases in median AFM1 levels were observed at 3 months after intervention ($p < 0.05$). While the median AFM1 level was comparable between the placebo (PL) group and the LD group, a reduction rate of 58.7 % in the median AFM1 level was found between the HD group and the PL group ($p < 0.05$). Due to the sorptive properties of the silicate it was able to bind the aflatoxin occurring in food within the digestive tract and thus withdraw it from the metabolism of the test subjects. A very wide range of AFM1 levels (from undetectable to 13.3 ng/mg creatinine) was observed in the study participants. The authors stated this may be attributed to seasonal changes in food contamination in the region, as well as genotypic or phenotypic variations in AF-metabolizing enzymes and individual susceptibility. Nevertheless, significant dose–time interaction effects associated with reduced urinary AFM1 levels in this study confirmed the efficacy of the clay test substance. No significant differences could be detected concerning hematological parameters, liver and kidney function or electrolyte concentration. Sporadically there were differences in the serum parameters, which nevertheless were within the normal physiological range and did not follow a dose-dependent trend. Side effects associated with the administration were not observed* [Afriyie-Gyawu et al., 2008; Phillips et al., 2008; Wang et al., 2008; Wang et al., 2005].

The route of administration (oral) and the mechanism of action (adsorption on a silicate that stays inside the digestive tract) are comparable to those described in animal studies with clinoptilolite* [Gallo and Masoero, 2010; Masoero et al., 2009; Bulut Albayrak, 2012; Grenier and Applegate, 2015; Oğuz et al., 2000b].

4.3.2 Animal Data

In a study by Ortatatli et al. [2005] chickens were administered a mixture of several aflatoxins (consisting mainly of aflatoxin B1) in feed (50 ppb or 100 ppb total aflatoxin) for 41 days. A test group of chickens received the contaminated feed as well as clinoptilolite supplementation at concentration of 1.5 % (w/w). Macroscopic changes could not be observed in any group, as the levels of AFB1 applied were relatively low, which is in agreement with observations of other authors conducting similar studies. Nevertheless, addition of clinoptilolite to the 100 ppb group partially but significantly decreased both the incidence of affected broilers and the severity of the lesions in the organs investigated. The beneficial effect of clinoptilolite in this study might be attributed to the high affinity of

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clinoptilolite for AFB1 and the resulting reduction in bioavailability of the dietary aflatoxins. Subsequently, AFB1 couldn't be adsorbed in the gastrointestinal tract and effects associated with bioavailable AFB1 were less pronounced* [Ortatatli et al., 2005].

Oguz/Oğuz et al. [2000a, 2000b] drew comparable results with studies regarding the effect of clinoptilolite on chickens. According to the authors, one approach to solve the problem of AF in the feed has been to use nonnutritive and inert adsorbents in the diet to bind AF and reduce their resorption into the gastrointestinal tract. These adsorbents must not be resorbed into the gastrointestinal tract and must have the ability to bind physically with chemical substances, precluding their resorption. Besides, clinoptilolite has been used as such an adsorbent in animal feeding. In the study described, clinoptilolite incorporated into the diet at 1.5 and 2.5 % was evaluated for its ability to bind AF and reduce the deleterious effects of 2.5 mg total aflatoxin (more than 75 % aflatoxin B1) per kg diet on broiler chickens from 1 to 21 days of age. In total 360 broiler chicks were divided into six equal treatment groups (control, AF, clinoptilolite (1.5 %), AF plus clinoptilolite (1.5 %), clinoptilolite (2.5 %) and AF plus clinoptilolite (2.5 %)). When compared to controls, AF treatment significantly decreased serum total protein, albumin, inorganic phosphorus, uric acid, total cholesterol, haematocrit, red blood cell counts, mean corpuscular volume, haemoglobin, thrombocyte counts and percentage of monocyte counts, and increased values of white blood cell and heterophil counts. The addition of clinoptilolite (1.5 %) to the AF-containing diet improved the decrease in thrombocyte counts caused by AF, most likely by decreasing the bioavailability of AF. Furthermore, by addition of clinoptilolite (1.5 %) to the AF-containing diet and subsequent reduction of the bioavailable AF, the AF-related changes in red blood cell counts, haematocrit, haemoglobin, white blood cell counts, percentage of lymphocytes and monocyte counts were intermediately ameliorated* [Oguz et al., 2000a, Oğuz et al., 2000b].

Safameher [2008] showed that changes of the serum parameters in chickens, caused by the addition of aflatoxins, are significantly reduced with the feeding of 2 % clinoptilolite. According to the author, clinoptilolite (CLN) as an inert sorbent reduces the resorption of dietary mycotoxins into the gastrointestinal tract. Besides, clinoptilolite is preferred as a binding agent due to its high binding capacity against AF and its reducing effect on AF resorption in the gastrointestinal tract. In the actual study, clinoptilolite was added to diets

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containing aflatoxins (AF) for 41 days. A total of 480, one-day-old broiler chicks (Ross 308) was divided into 6 treatments, each consisting of 80 chicks: control; basal diet prepared with uncontaminated diet, diet containing AF (0.5 mg/kg), diet containing AF (1 mg/kg), diet containing clinoptilolite (20 g/kg), diet containing AF (0.5 mg/kg) + clinoptilolite (20 g/kg) and diet containing AF (1 mg/kg) + clinoptilolite (20 g/kg). Chickens were monitored daily and then body weight and feed consumption were recorded. Plasma enzymatic and non-enzymatic parameters showed progressive changes in birds examined 21 or 42 days after dietary aflatoxin treatments. Compared to controls, the AF treatment significantly ($p < 0.05$) decreased body weight gain, feed intake, serum total protein, albumin, cholesterol, uric acid, calcium, phosphorus, and alkaline phosphatase (ALP), while the enzymatic parameters lactate dehydrogenase (LDH) and aspartate amino transferase (AST) increased. Decreases in serum parameters caused by AF was significantly ($p < 0.05$) ameliorated by clinoptilolite. A similar increase was obtained in feed intake and body weight gain by adding clinoptilolite to the AF-containing diet ($p < 0.05$). The addition of clinoptilolite to the AF-containing diet decreased the severity of negative effects of adsorbed dietary AF. The results suggest that clinoptilolite positively influenced several parameters due to aflatoxin not entering the body but being bound to clinoptilolite in the digestive tract* [Safameher, 2008].

Milk is prone to aflatoxin contamination as there is a carry-over from feed to milk at a rate of 1 – 6 %. Katsoulos et al. [2016] showed under field conditions that by adding 1 % (w/w) of clinoptilolite to feed of dairy cattle for a period of seven days, there is a clear and significant reduction of the aflatoxin level in the milk produced. Fifteen commercial Greek dairy herds with AFM1 concentrations in bulk tank milk ≥ 0.05 µg/kg (caused by consuming feed rations naturally contaminated with AFM1) were selected. Participating farms had to meet the inclusion criteria for the difference between bulk tank milk AFM1 concentration at the initial measurement and at the onset of the experiment (3 – 10 days later) not being more than 10 %. Bulk tank milk AFM1 was determined prior to the onset and on day 7 of the experiment. The duration of 7 days was considered to be an adequate study period given that it is about double the clearance time after the removal of aflatoxins of the dairy cattle diet which is about 3 – 4 days. Clinoptilolite was added in the total mixed rations of all farms at

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the rate of 200 g per animal per day, throughout this period. Besides the addition of clinoptilolite, rations remained unchanged during the whole experiment. Two different particle sizes of clinoptilolite were used: one was below 150 µm and another below 800 µm (mean particle size of G-PUR® is 3 µm). Especially for the finer clinoptilolite particle size, there was a linear correlation between the aflatoxin levels before and after 7 days of continuous clinoptilolite application. For the 15 different farms that were included in the study, the mean aflatoxin level reduction in milk by addition of clinoptilolite to the feed for one week was more than 50 % of the initial aflatoxin level before clinoptilolite administration and reached a statistical significance of $p < 0.001$. Furthermore, there was a significant and strong linear correlation among the milk AFM1 concentrations on days 0 and 7 ($R = 0.95$, $P < 0.001$, $r^2 = 0.899$). The authors state that the results obtained prove efficacy of dietary administration of clinoptilolite under field conditions and that clinoptilolite is effective in reducing the milk concentration of AFM1. The criteria of farm selection were set in order to ensure, as much as possible, that the milk AFM1 concentration on day 0 represents a stable situation of high milk AFM1 and that the reductions detected on day 7 are due to the infeed inclusion of clinoptilolite* [Katsoulos et al., 2016].

The average relative reduction of milk AFM1 recorded in this study, although not comparable with other experiments due to different study design, was similar to those achieved with other alumosilicate mycotoxin binders; Diaz et al. [2004] observed that calcium and sodium bentonite products reduce milk AFM1 concentrations by 31 % to 65 %, most likely due to reduction of bioavailability. Kutz et al. [2009] observed that two hydrated sodium calcium alumosilicates decreased the AFM1 in milk by 45 and 48 %* [Diaz et al., 2004; Kutz et al., 2009].

This may also be used as additional evidence that studies, which used other silicates like bentonite or hydrated sodium calcium alumosilicates (HSCAS), may also be applicable for clinoptilolite, as the mechanism of action is comparable, as both substances are quite inert and show a high affinity for AF*.

Furthermore, it clearly indicates that finer particle sizes are more effective in adsorbing dietary aflatoxins than coarser particle sizes. A smaller particle size provides a larger surface that is available for interaction with the polar mycotoxins such as aflatoxins [Papaioannou et al., 2005]. Particle sizes used in the study of Katsoulos et al.

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[2016] were in a range below 150 µm and 800 µm, whereas G-PUR® has a mean particle size of only 3 µm, suggesting even higher efficacy in binding dietary aflatoxins than the particle sizes used by Katsoulos et al [2016] *.

In a study by Parlat et al. [1999], Japanese quail chicks from 10 to 45 days of age received a diet fortified with aflatoxins (mainly consisting of AFB1 and AFB2) and supplemented with clinoptilolite (when various poultry species were fed diets with different concentrations of aflatoxins, poults and goslings appeared to be the most sensitive, quail were most vulnerable, and domestic chicks were most resistant). The experimental design consisted of 4 dietary treatments: basal diet; basal diet plus 2 mg AF/kg diet; basal diet plus 50 g clinoptilolite, and basal diet plus 2 mg AF plus 50 g clinoptilolite/kg diet. Basal diet consisted mainly of maize, barley, soybean meal, sunflower seed meal, fishmeal and vegetable oil. Researchers reported that cumulative food consumption was reduced by 14 % among the quail chicks fed on the AF-containing diet, however the reduction was only 6 % for chicks consuming the AF plus clinoptilolite diet. Similarly, overall body weight gain was reduced by 27 % in the AF-containing group, but by only 8 % in the AF plus clinoptilolite group. In the study, a significant improvement was observed in all of the investigated performance parameters when comparing the group consuming the AF diet with the group consuming the AF plus clinoptilolite diet ($P < 0.05$) *.

The authors of the publication from Parlat et al. [1999] stated their observations suggest the addition of clinoptilolite as an adsorbent to the diet may be an encouraging approach to overcome the problems associated with aflatoxin effects in poultry. Clinoptilolite binds the AF and reduces their resorption in the gastrointestinal tract. Adsorbents like clinoptilolite, not being resorbed in the gastrointestinal tract, must have the ability to bind physically with aflatoxins, precluding their absorption. Zeolites have been used for this purpose. These are generally inert and non-toxic to animals and have a capacity to bind aflatoxins. This study suggests that – even for very high concentrations of aflatoxins – clinoptilolite is able to reduce the bioavailability of dietary AF, even in a complex matrix like the chicken diet applied, which consisted of many ingredients that can also be found in a complex human diet* [Parlat et al., 1999].

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4.3.3 Lab Data

For most regulated mycotoxins, G-SCIENCE®. Inc. performed *in-vitro* experiments to provide data for the specific toxin-clinoptilolite interaction. The adsorption capacity of G-PUR® for the relevant toxins was determined in 3 different matrices, starting with a good solvent for the toxin (methanol 70 %), water or physiological saline solution, and simulated gastric juice. Depending on the type of toxin, particular strong interactions between G-PUR® and the toxin could be detected*.

mycotoxin	interaction with Clinoptilolite
Aflatoxin B1	+++
Zearalenone	+
T2-Toxin	+
Citrinin	++
Fumonisin B1	+++
Ochtratoxin A	+
Deoxynivalenol	-

Table 6 *Interaction of G-PUR® with various mycotoxins in-vitro*
[G-SCIENCE®, Inc., unpublished]

These results are in line with relevant scientific publications:

Dakovic et al. [2000] analyzed the interaction between aflatoxin B1 and G2, and clinoptilolite. Particle size of clinoptilolite used was below 63 µm. Kinetics of aflatoxin B1 and G2 adsorption were analyzed at pH 2 and pH 7, with a time interval of 5 min to 48 h. The composition of the electrolytes in the study material was similar to that of gastric juice in animals, and the temperature was 37 °C. For both toxins, the authors observed a rapid adsorption process, with most of the toxin being adsorbed within the first few minutes. In addition, adsorption was characterized by the authors to be almost irreversible. For all experiments, at different pH levels and aflatoxin concentrations, adsorption of aflatoxins G2 and B1 on clinoptilolite was always greater than 80 %* [Dakovic et al., 2000].

In a study by Lemke et al. [2001] several adsorbents were tested for their aflatoxin binding in a gastro-intestinal (GI) experimental model with 2 hours incubation at pH 1.3, followed by 2 hours at pH 7 with pancreatin and bile salts. Clinoptilolite was able to bind about 40 % of the available aflatoxin B1 in a simulated GI fluid (clinoptilolite bound 7 mM/kg in a setup with 5 ml of a 8 µg/ml AFB1 solution and 1 mg clinoptilolite). More than 50 % of the bound aflatoxin could not be recovered even by organic solvents (methanol and chloroform), as the adsorption strength of the clinoptilolite-aflatoxin complex is very high* [Lemke et al., 2001].

This further substantiates the hypothesis that the aflatoxin-clinoptilolite complex is of extraordinary

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stability, not releasing the bound aflatoxin despite of the matrix *.

Bočarov-Stančić et al. [2011] have used an *in vitro* model for the evaluation of the binding capabilities of several natural binders and six different mycotoxins. For the adsorption experiments the ability to bind mycotoxins was evaluated in a buffered electrolyte solution at different pH levels. Concentration of the mycotoxins ranged from 0.2 to 2 mg/l. Though a relatively course particle size has been used compared to G-PUR® (d50 ~ 13 µm compared to G-PUR® with 3 µm), most of the mycotoxins analyzed could be adsorbed by clinoptilolite. Only ochratoxin A and diacetoxyscirpenol did not interact with clinoptilolite at neither pH* [Bočarov-Stančić et al., 2011].

In a study of Vekiru et al. [2015] "intensified conditions" were used to analyze the efficacy of several sorbents. In an *in-vitro* experiment, a very high concentration of aflatoxin B1 (4 mg/l) in different liquid matrices (buffer or gastric juice) was put into contact with relatively low (0.02 %) concentrations of different adsorbents. Even in these extreme conditions, there still was an interaction between clinoptilolite and aflatoxin B1, adsorbing between 6.4 % and 21.9 % of the toxin. In a second experiment, broiler chicks were exposed to spiked feed (at an aflatoxin concentration of 2 mg per kg feed) and different adsorbents at 0.5 % (w/w). In this setup, clinoptilolite was not effective to impact the effects of aflatoxin B1, what might be due to the fact that the clinoptilolite concentration was quite low and aflatoxin concentration was extremely high* [Vekiru et al., 2015].

Tomasevic-Canovic et al. [2001] evaluated the affinity of different cation-exchanged forms of clinoptilolite for aflatoxin B1 *in vitro*. The solution from which the adsorption was to be examined was chosen to simulate the gastric juice of animals and contained: 0.1 mol/dm^3 HCl and 0.05 mol/dm^3 NaCl. The experiment was carried out at pH 3.8 and at a temperature of 37 °C. A certain amount of AFB1 (200 µg) was added to 100 cm^3 electrolyte and an aliquot (0.4 cm^3) was taken for the determination of the total toxin concentration present in the solution. Then, 1 g of mineral adsorbent was added to the contaminated electrolyte solution. At the end of the reaction time (2 h), the concentration of non-adsorbed AFB1 was determined in the supernatant. Natural and Ca-rich clinoptilolite showed a very high chemisorption index comparable to the montmorillonite-clay analysed. The authors concluded that clinoptilolite adsorbed a substantial amount of aflatoxin B1 and the presented *in vitro* data clearly demonstrate that,

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at a concentration of 200 µg per g of adsorbent, all of the mineral adsorbents used greatly reduce the bioavailability of AFB1. Ratio of AFB1 and adsorbent correspond to 1 mg/kg of AFB1 in the diet, if the adsorbent is incorporated into the diet at a level of 0.5 %* [Tomasevic-Canovic et al., 2001].

The totality of evidence demonstrated in the numerous scientific and peer reviewed publications, together with the G-PUR® lab data and the human study published by Afriyie-Gyawu et al. [2008]; Phillips et al. [2008]; Wang et al. [2008]; Wang et al. [2005] clearly suggests that alumosilicates like clinoptilolite bind and reduce the bioavailability of dietary mycotoxins*.

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5 Data and Supportive Information to Determine the Safety of G-PUR®

5.1 Comprehensive Safety Profile for G-PUR®

Data and information relevant to the safety of G-PUR® (purified clinoptilolite) were obtained from product specific toxicity studies, and – where applicable – published studies identified during a comprehensive review of the scientific literature. Studies were selected from the literature search on the basis of their relevance to the safety of G-PUR®. They were also selected based upon quantitative and qualitative similarity of the test material studied in relation to the chemical composition of G-PUR® and in consideration of scientifically sound study methodologies conducted in accordance with international standards.

G-PUR® is not soluble in water, and it is generally accepted that tectosilicate structures with high silicon to aluminum ratios are highly stable to acid degradation. No loss in crystal structure (as determined using X-ray diffraction, energy dispersion-X-ray fluorescence, and transmission electron microscopy) and no change in metal composition of the mineral (as determined using inductively coupled plasma mass spectrometry (ICP-MS)) were observed at pH values ≥ 2 [Li et al., 2008].

In vivo studies using porcine models (FDA considers pigs to be the most appropriate animal model for the human digestive tracts [U.S. FDA, 2011]) have demonstrated that dissolution of the alumosilicate structure of clinoptilolite will not occur during gastro-intestinal passage in humans, as no evidence of aluminum bioavailability can be identified in pigs administered clinoptilolite in the feed at significant dietary levels of up to 2 % [Pond et al., 1989; Papaioannou et al., 2005; Alexopoulos et al., 2007].

The high stability of clinoptilolite minerals to acid digestion are in contrast to other silicates (e.g. synthetic Zeolite A), which are subject to partial dissolution at the physiological pH of the stomach (pH ~2). Silicate minerals such as synthetic Zeolite A have an aluminum:silicon ratio of approximately 1:1, and the higher concentration of low-energy aluminum-silicon bonding throughout the crystal structure of these minerals renders these minerals susceptible to acid hydrolysis. These differences in the chemical stability of clinoptilolite and other silicates are relevant to the safety assessment of G-PUR® as toxicity studies conducted in rodents and dogs administered various chemically related silicates (i.e. Zeolite A) have identified lesions in the kidneys and bladder [HERA, 2004; JECFA - WHO FAS No. 5, 1974; Cefali et al., 1995].

These lesions can be identified within 90 days of treatment and are associated with accumulation of silicon in the kidneys and presence of non-

staining crystalline material deposited in the renal pelvis and urine, an observation that is attributed to resorption of small amounts of silicon-compounds in the gastrointestinal tract after dissociation of sodium aluminum silicate to sodium, aluminum and SiO_4 [HERA, 2004]. Accordingly, observations of bladder and kidney toxicity in animals administered silicate compounds with a low silicon to aluminum ratio (i.e. Zeolite A) were not considered relevant to the safety of G-PUR®.

Clinoptilolite minerals are permitted for consumption in the European Union under EC Directive 93/42/EEC, and the current and historical consumption patterns of these products in the European marketplace also were considered relevant to the safety determination. These products are authorized for consumption at use levels between 2 to 10 g per day for repeated daily consumption by the general population. Comprehensive chemical analyses of clinoptilolite products legally permitted for sale in the European marketplace since 2006 were conducted using X-ray diffraction and ICP-MS. A comparison of the X-ray diffraction profiles and elemental composition of G-PUR® to other clinoptilolites authorized for consumption under EC Directive 93/42/EEC demonstrated these products were qualitatively and quantitatively comparable to G-PUR® raw material, and in fact have a very high probability of sharing the same geological origin for sourcing of the raw material.

Findings from controlled studies identified in the literature evaluating the dietary consumption of Enterex, a natural clinoptilolite mineral consumed in the EU, were reviewed for potential reports of adverse effects. Short-term studies conducted in over 500 subjects administered Enterex at levels of up to 10 g per day did not result in notable test article related adverse effects of clinical significance [Rodriguez-Fuentes et al., 1997]. G-SCIENCE®, Inc. has conducted comprehensive studies comparing the qualitative and quantitative chemical composition of clinoptilolite minerals originating from the Tasajeras deposit in Cuba (source of clinoptilolite used in Enterex) and have demonstrated that the source material is chemically similar to G-PUR® raw material.

5.2 Patented Purification Process

Certain contaminants that are always present in natural clinoptilolite (despite its origin) may pose a significant risk if applied to humans.

In a study with weaned pigs, Fokas et al. [2004] determined the retention coefficient of Pb from a natural, not purified clinoptilolite of Greek origin. Twelve pigs were divided into two groups, one group received a diet that contained 2 % of a Greek clinoptilolite that contained elevated amounts of Pb (46 ppm). The authors found that

liver lead content significantly increased in the group that received the natural clinoptilolite of Greek origin, though other organs did not show a statistically significant change [Fokas et al., 2004].

The study performed by Petrakakis et al. [2007] clearly showed that lead becomes available and may be leached from clinoptilolite at certain pH levels. In batch reactor experiments with pH and temperature within physiological ranges, significant amounts of lead could be leached from clinoptilolite [Petrakakis et al., 2007].

This clearly emphasizes the necessity to carefully choose the specific source from which clinoptilolite is mined, and the need for a purification process that minimizes all contaminants to the lowest possible concentrations. G-SCIENCE®, Inc. understands the value and challenges inherent with clinoptilolite and developed a proprietary process to remove natural contaminants from G-PUR® to ensure it is both safe and efficacious [Glock, 2006].

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