Use of supercritical CO_2 for bone delipidation

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Supercritical carbon dioxide was used for bone delipidation. It appeared that this technology is very efficient since supercritical CO_2 is able to diffuse into microporous solids much better than liquids and that it has a good solvent capacity for lipids. This extraction is the ideal first step of any bone processing because microporosity of bone tissue becomes much more accessible, which may enhance osteoconduction once implanted. Moreover, it is safe since it involves no toxic chemical and is potentially usable with allografts as well as xenografts. *Biomaterials* (1994) **15**, (9) 650–656

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Above a critical pressure (P_c) and a critical temperature (T_c), pure components are no longer gaseous or liquid. They are in another state called supercritical fluid. *Figure 1* shows a P, T diagram in which the supercritical domain has been delimited by dashes. Due to their unique properties of diffusivity, density and viscosity, supercritical fluids (SCFs) are used as selective extraction solvents[1]. Like liquids, they have a high density and consequently a high solvent capacity which is, to a first approximation, directly dependent on the density. The most remarkable property of SCFs is that they exhibit a wide spectrum of solvent capacities. It is possible to fine tune this capacity just by varying the working pressure or temperature.

However, the other physicochemical properties of SCFs are of great interest. In particular, they display gas-like transport properties because of their very low viscosity and very high diffusion coefficients. Therefore, they are particularly adapted to the extraction of components entrapped in a solid microporous matrix, as is the case with bone.

In most of the processes described in the literature, CO_2 is the most used solvent because it is natural, safe (non-toxic, non-corrosive, non-flammable), easily available and cheap. Moreover, its mild critical coordinates (critical pressure 7.38 MPa; critical temperature 31°C) are relatively easy to reach and are compatible with biological compounds. Its use implies the total absence of solvent residue and off-odours, since it returns to the gaseous state at atmospheric pressure. Non-polar components like hydrocarbons, oils and generally all lipids are soluble in supercritical CO_2. Several data about lipid solubility in supercritical CO_2 have been published[2–4]. On the other hand, polar molecules, amino acids and proteins are much less soluble.

The use of supercritical solvents has been developed mainly in the oil industry and the food industry for the extraction of petroleum[5] and fossil fuels, coffee decaffeination[6] and extraction of vegetal or animal materials.

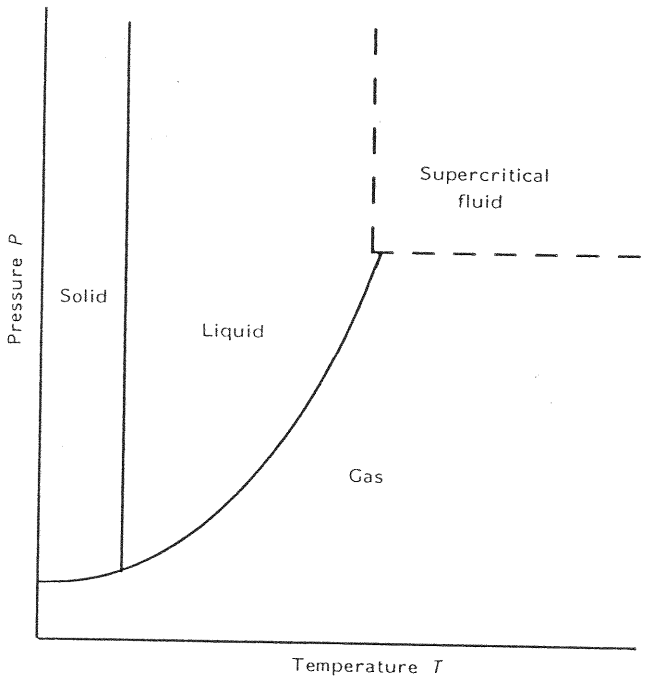

Figure 1 Pressure–temperature diagram for a pure component.

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Although their properties have been known for more than a century (Cagniard de la Tour, a Frenchman, discovered their existence in 1822), their use is still considered as a new technology since the interest of researchers focussed on them about 20 yr ago and the major industrial applications emerged in the mid-seventies. Their field of application has recently extended to the cosmetic and pharmaceutical industries[1]. However, this technology, up to now, has not been used in the biomedical industry. This paper is the first description of the use of supercritical CO_2 for bone tissue treatment.

The use of bone tissue substitution material is developing with the increasing number of allografts (human bones) and xenografts (animal bones) carried out daily by orthopaedic surgeons all over the world. The development of allografts with bones coming from bone banks has revealed a serious infection problem. This problem is linked with AIDS development, since several cases of HIV and other virus transmission by frozen allografts have recently been described and isolation of HIV from different bone samples has been found[7,8]. In addition, adverse immunological responses have been reported and appear to be still poorly understood. Moreover, recolonization of implants by new bone tissue is often poor[9]. The use of animal bone tissue has also been extensively tested. In comparison with human bones it exhibits two major advantages: it is almost freely available and it avoids the ethical problems of tissue retrieval from humans. The immunological response is present but was generally judged as weak[10], provided all potentially antigenic constituents have been removed. To minimize this response it is necessary to eliminate medullar tissues which are responsible for immunogenic reactions. The defatting process is the first step of animal bone processing and is crucial for good osseointegration of the graft, since the osteoconduction is directly correlated with the wettability of the graft. Specific or extensive deproteination generally follows this defatting step[11]. These two operations are generally carried out with chemical organic solvents. The most used have been ethylenediamine, hydrogen peroxide, and several chlorinated solvents such as chloroform or dichloromethane. Ethanol and acetone have often been used as complementary solvents.

However, the microporous nature of bone tissue implies that:

1. problems of wettability prevent accessibility of micropores to the solvents used;
2. adsorption of solvent molecules at the inner surface of the pores is important.

Therefore two major drawbacks of these classical procedures arose:

1. potentially antigenic determinants are not removed by the process;
2. toxic residues which cannot be removed by washing remain after processing.

This is a possible explanation for the controversial use of such implants, which have been considered as unsatisfactory.

In this paper, the characterization of the biomaterial resulting from bovine bone tissue treatment by supercritical CO_2 is described, the advantages and drawbacks of such a treatment are presented and the possible prospective utilization of this new application is discussed.

MATERIALS AND METHODS

Bones

We used bovine femur condyles and femoral heads. Animals came from abattoirs with EEC agreements and were killed for human consumption. After removal of all soft tissues, parallelepipeds of size approximately 15 × 15 × 20 mm were cut using a band saw in the cancellous part of the bone.

Supercritical extraction device

The pilot plant (Separex, Champigneulles, France) for continuous extraction and separation was located at the biochemical engineering department at the 'Institut National des Sciences Appliquées' in Toulouse (*Figure 2*). In this apparatus, cooled liquid carbon dioxide (1, 2) is pressurized by a metallic membrane pump (3) (Dosapro-Milton Roy, Pont Saint, Pierre, France) and then heated to the extraction temperature (5). The extraction vessel, with a volume of 200 ml (6), is followed by a series of four thermostatically controlled (9) separation vessels (7), in which pressure is adjusted by needle valves (8). At the outflow of the fourth separator, CO_2 is liquified (2) and recycled. At the bottom of each separator, a discontinuous recovery of the condensed liquid phase is possible. A mass flow meter (10), based on the Coriolis effect, was used to determine mass flow rate. Operating conditions were defined accordingly to Fages *et al.*[12].

Proteic extraction

We used two different processes: hydrogen peroxide maceration at 40°C for 12 h and protease maceration (Alcalase, Novo) at 50°C for 24 h.

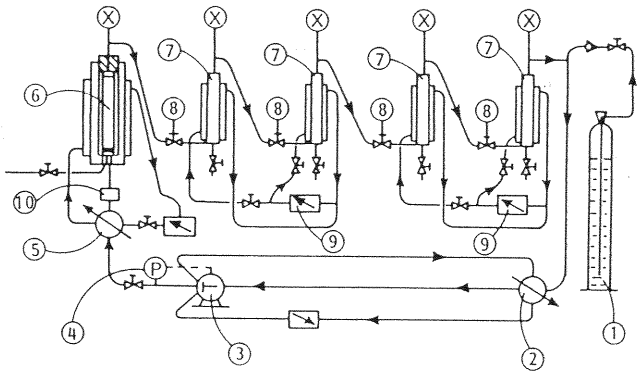

Figure 2 Scheme of the supercritical pilot extraction device. 1, Liquid CO_2 tank; 2, cooler; 3, high pressure pump; 4, pressure controller; 5, heater; 6, extraction vessel; 7, separation vessels; 8, needle valves; 9, heater; 10, mass flowmeter; ×, temperature and pressure measurements and security valves.

Mechanical properties

Compression strength was measured using a Nene testing system.

X-ray diffraction analyses

These were performed with a Philips diffractometer using Co-$K\alpha_1$ radiation (λCo-$K\alpha_1$ = 17.8892 nm).

Infrared analysis

These were achieved with a Perkin–Elmer 1600 series FTIR device. Samples were mixed with KBr (1.5 mg sample/300 mg KBr) and pressed into thin discs.

Electron microscopy analysis

This was done at the University of Toulouse (Faculty of Medicine). The samples were prepared as follows: bone cells cultured on hydroxyapatite (HA) crystals as described by Frayssinet *et al.*[13] were exposed to supercritical CO_2 flow under the same conditions as those used with bone samples. Bone cells on HA and bone samples were dehydrated with several baths of increasing ethanol concentrations, then with acetone and coated under vacuum with gold–palladium. They were observed at 25 kV using a Jeol JST200 scanning electron microscope.

Chemical analyses

These were performed by Europe Sols (Toulouse, France) on crushed samples. Nitrogen analysis was by the Kjeldhal method, organic carbon (OC) by an oxido-reduction method and lipid (L) analysis by ethyl ether extraction.

Thermogravimetric analyses

These were performed at the Laboratoire de Physico-chimie of the Institut National Polytechnique (Toulouse, France) using a SETARAM (Lyon, France) microscale MTB10-8 at a rate of 6 °C/min.

Organic matter content

The organic matter content (OM) was calculated in three different ways.

1. From ash content (AC) following calcination at 950 °C. For the calculation, the water content (W) and the percentage of CO_3^{2-} ions in the apatite [CO_3] was determined from thermogravimetric experiments. Their average values, respectively $W = 0.12P_0$ and $[CO_3] = 0.075AC$, were used for all the calculations. P_0 is the bone weight sample just before calcination. $OM_1 = \{P_0 - ([CO_3] + W + AC)\}/P_0 \times 100$.
2. From nitrogen analysis, assuming that proteinaceous organic matter has a nitrogen content of 15.8% (w/w), which is the value of the nitrogen content of type I collagen, which accounts for about 90–95% of the bone organic phase[14]. The total organic content is assumed to be equal to the sum of the proteinaceous organic matter content and the lipidic fraction (L), $OM_2 = (N/0.158) + L$.
3. From organic carbon analysis. The carbon content of the proteinaceous fraction is equal to the total organic carbon content (TC) minus the amount of carbon from the lipidic fraction (LC). Assuming that proteinaceous organic matter has a carbon content of 42.0% (w/w), which is the value of the carbon content of the type I collagen, and that the lipidic fraction has a carbon content of 75%, the total organic content is assumed to be equal to the sum of the proteinaceous organic matter content and the lipidic fraction, $OM_3 = ((TC - LC)/0.420) + L$.

The values of nitrogen and carbon content of type I collagen were calculated from Nimni[15], who gave the percentage of amino acid residues per 1000 total amino acid residues for α_1 and α_2 chains of type I collagen, which is composed of two α_1 chains for one α_2 chain.

Statistical analysis

Ninety-five percent confidence intervals (IC) of the mean were calculated using the formula:

$$IC = \sigma_{n-1} \cdot \frac{t_{(n-1;0.975)}}{\sqrt{n}}$$

where n is the sample size, $t_{(n-1;0.975)}$ is the 97.5 percentile of the Student's t distribution with $n - 1$ degrees of freedom and σ_{n-1} is the sample standard deviation.

RESULTS AND DISCUSSION

Qualitative study

Electron microscopy analysis

To determine whether non-lipid fractions of the medullar tissues are removed or not by the supercritical extraction, we performed scanning electron microscopy analysis on two types of samples.

1. Bone cells cultured on HA crystals, exposed at the supercritical CO_2 flow. As shown in *Figure 3*, the cells appeared unchanged after the treatment. This is a confirmation that CO_2 extraction does not extract cellular components.
2. Bone tissue samples after CO_2 extraction exhibited cellular debris on their surface (not shown). On the other hand, bone tissue samples after CO_2 extraction and an additional extraction step (either chemical or enzymatic) completely eliminated this debris and the bone surface appeared perfectly clean. *Figure 4* shows a completely empty osteoplast.

Infrared analyses

FTIR spectra of both one-step and two-step extracted bone tissue are very similar and give a qualitative confirmation that the structure of the bone tissue remained unchanged. *Figure 5* gives an example of a spectrum of a double-extracted sample. Absorption bands at 600 and 1000 cm^{-1} are characteristic of apatite orthophosphoric ions. The presence of CO_3^{2-} ions in the apatitic structure is evidenced by the absorption bands at 870, 1420 and 1470 cm^{-1}. A Hydroxyl group absorption band of HA occurs at 3600 cm^{-1}. Collagen C–H bands form shoulders

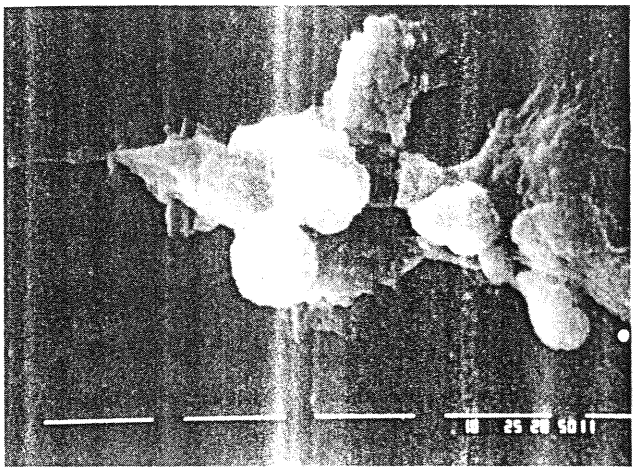

Figure 3 Bone cells cultured on hydroxyapatite after being exposed to supercritical CO_2. Scanning electron microscopy. Bar = 10 μm.

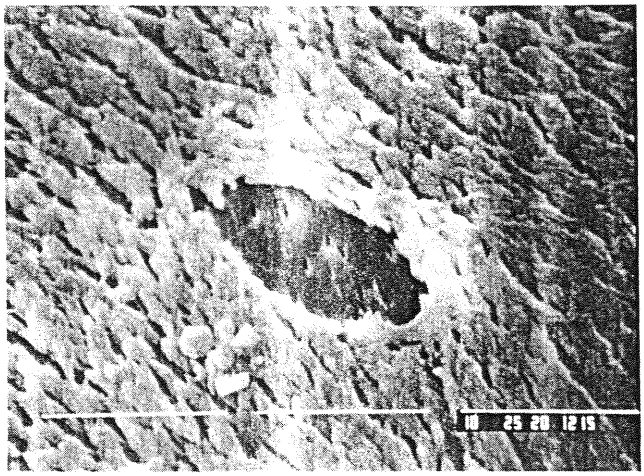

Figure 4 Empty osteoplast at the bone tissue surface after supercritical CO_2 extraction and H_2O_2 extraction. Scanning electron microscopy. Bar = 10 μm.

between 2800 and 3000 cm^{-1} in the broad envelope at 2700–3400 cm^{-1}, which is due to the elongation mode of the O–H link of the structure water molecules. Several bands in the range 1500–1700 cm^{-1} can be attributed to the absorption of C–O, C–H and N–H links of protein functional groups.

X-ray diffraction analyses

X-ray radiodiffractograms after one or two extraction steps (*Figure 6*) are very similar to that of native bone. The mineral phase is composed of poorly crystalline apatite, as evidenced by the 002 peak and the envelope of the 211-112-300 lines which are characteristics of the apatitic crystalline network[16].

Quantitative study

Characterization of the untreated cancellous bone tissue

Analysis results are shown in *Table 1*. It is noteworthy that most of the organic matter of the untreated bone is

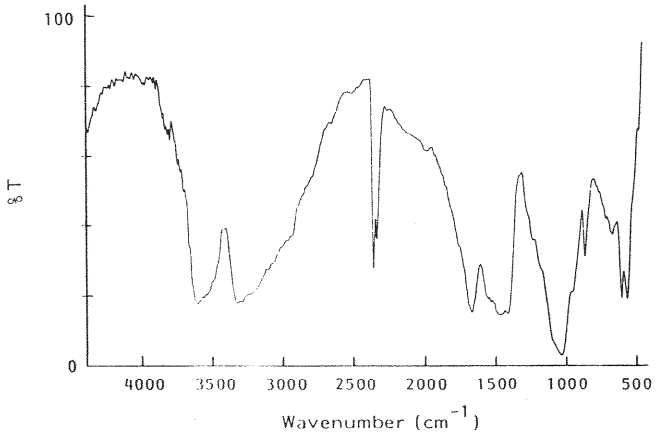

Figure 5 Infrared absorption spectrum of bovine cancellous bone after supercritical CO_2 extraction and H_2O_2 extraction.

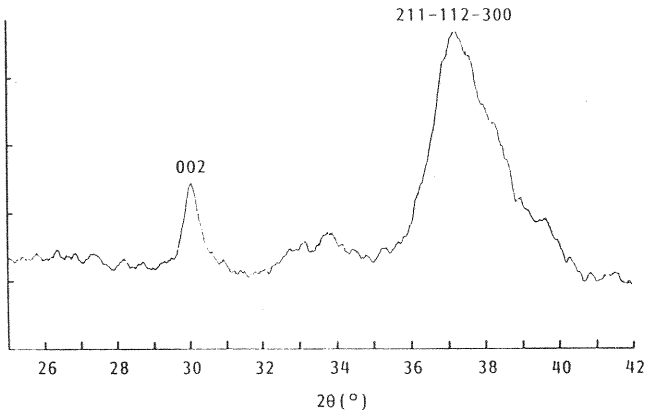

Figure 6 X-ray diffraction profile of bovine cancellous bone after supercritical CO_2 extraction and H_2O_2 extraction.

composed of medullar tissues. The relative part of these tissues will be evaluated in the next paragraph. Untreated bone parallelepipeds are very heterogeneous and therefore the chemical analyses done on an aliquot are very unprecise. The lipid content is very likely underestimated (see next paragraph) and this may explain both the underestimated value of OM_2 and the overestimated value of organic matter OM_3.

Characterization of the bone tissue and the extract after the supercritical fluid extraction step

As shown in *Table 2*, the extract which is almost nitrogen free is quasi-exclusively composed of fats, which are known to be the main constituent of medullar tissues. However, the cellular debris and other non-lipidic components present in this tissue are not extracted, which is in accordance with what is known on the solvent capacity of supercritical CO_2 (see paragraph on scanning electron microscopy).

On average, the extract accounted for 51.3% of the initial weight of the bone samples. Therefore the lipid content of the fresh bone samples is $0.513 \times 0.979 = 50.2\%$.

Assuming that the non-extracted part of the medullar tissue can be neglected (in terms of weight), we can postulate that the composition of the bone tissue after

Table 1 Characterization of the untreated cancellous bone tissue

Nitrogen content	Organic carbon content	Lipid content	Ash content	OM_1	OM_2	OM_3
2.36 ± 0.10	46.56 ± 1.99	45.70 ± 6.11	23.8 ± 1.8	62.4 ± 1.98	60.6	75.0

Results are in % (w/w), followed by ± confidence interval of the mean.

Table 2 Characterization of the bone tissue and the extract after the supercritical fluid extraction step

	Nitrogen content	Organic carbon content	Lipid content	Ash content	OM_1	OM_2	OM_3
Bone	4.24 ± 0.29	11.75 ± 0.88	1.48 ± 0.63	55.4 ± 1.3	28.4 ± 1.4	28.3	26.7
Extract	0.06	n.d.	97.9	n.d.	n.d.	n.d.	n.d.

Results are in % of the bone weight just before treatment, followed by ± confidence interval of the mean. n.d., not determined

Table 3 Characterization of the bone tissue after the additional extraction step

Additional extraction step	Nitrogen content	Organic carbon content	Lipid content	Ash content	OM_1	OM_2	OM_3
H_2O_2	3.86 ± 0.08	8.85 ± 0.14	0.50 ± 0.09	63.5 ± 0.6	19.72 ± 0.52	24.9	20.7
Enzymatic	4.08 ± 0.23	9.58 ± 0.20	1.27 ± 0.35	62.6 ± 0.5	20.71 ± 0.58	27.0	21.8

Results are in % of the bone weight just before treatment, followed by ± confidence interval of the mean.

this first extraction step is that of the bone tissue itself. The bone composition as expressed in *Table 2* is in very good agreement with the results of Gong *et al.*[17], who found the following composition for trabecular bovine bone tissue: water, 14.5%; organic phase, 26.6%; CO_3^{2-} ions, 3.7%; ash content, 55.2%.

Characterization of the bone tissue after the additional extraction step

Results in *Table 3* indicate that both additional extraction processes lowered organic matter content through protein extraction as confirmed by the lowering of the nitrogen content. However, the H_2O_2 appears to be the most efficient since the nitrogen content, the organic carbon content and the lipid content are significantly lower in comparison with the enzymatic extraction step. Non-collagenous proteins, as well as cellular debris and other non-lipidic remnants of medullar tissue, are eliminated during this second extraction step.

Comparative thermogravimetric study of bone tissue after CO_2 extraction alone and after CO_2 and H_2O_2 extractions

The thermogram (*Figure 7*) shows the temperature decomposition of bone tissue after CO_2 extraction alone and after CO_2 and H_2O_2 extractions. This last curve is similar to that of bone tissue after CO_2 and protease extractions which is not shown in *Figure 7*. Both samples lost weight in three major steps.

1. Between ambient temperature and about 200°C: loss of 10–12% which can be attributed to water elimination. Both mineral and organic phase of bone tissue are known for their properties of water adsorption[18]. The extraction processes do not change these properties and the amount eliminated during this first stage is very similar to that of untreated bone tissue.
2. From 200 to 500°C: bone tissue after two extractions exhibits a loss of 20–23% of the initial weight sample. It is the organic matrix which is eliminated. The derivative curve shows a maximum weight loss speed at 330°C which is more than 15°C less than the maximum observed for the single extracted sample (346°C), which is the value described for bone tissue. This is confirmation that a fraction of the bone organic matrix (very likely the non-collagenous proteins) is eliminated by the second extraction step. This weight loss includes 30% of the carbonate ions of the mineral phase, which are eliminated between 200 and 300°C[19]. The second peak of organic matter loss corresponds at least partly to the non-lipidic fraction of the medullar tissue. This loss occurred at about 400°C for the double-extracted bone tissue sample and is obviously much less pronounced in comparison with bone tissue after CO_2 extraction. It is noteworthy that here again a shift of about 30°C has been observed.
3. From 500 to 900°C: loss of about 3% of the initial weight for both samples. The loss of the remaining

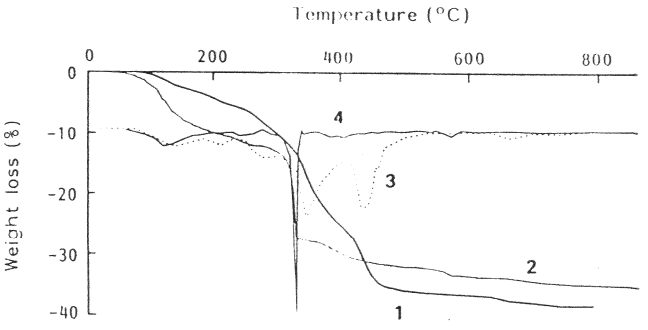

Figure 7 Thermogram of bovine cancellous bone after supercritical CO_2 extraction alone, **1**, and after supercritical CO_2 extraction plus H_2O_2 extraction, **2**. Curves **3** and **4** are differentials of curves **1** and **2**, respectively.

CO_3^{2-} ions occurred in this third stage. After this calcination we checked by X-ray diffraction and IR spectroscopy that we had pure recrystallized HA.

Organic matter determination

The organic matter content of bone tissue after supercritical CO_2 extraction has been found to be about 28% when calculated from calcination experiments (OM_1 in *Table 2*). Chemical analyses lead to very similar values of 27–28% (OM_2 and OM_3). The same types of results have been found for bone tissue after two extraction steps (see *Table 3*), although OM_2 values were slightly higher.

Thermograms of the different bone tissues give clear confirmation that the real value of organic matter content is very close to OM_1.

In order to confirm that the nitrogen content of the bone proteinaceous phase (15.8%) was not underestimated, we performed chemical analysis of completely demineralized bone tissue. On average, the analyses gave a C/N ratio of 2.47, suggesting that nitrogen content is higher than in type I collagen, which has a C/N ratio of 2.66. Therefore, the nitrogen content of the proteinaceous fraction of bovine cancellous bone tissue would be 17%. This may explain variations of OM_2 values of about 2%

Mechanical properties

After CO_2 extraction, the compressive strength has increased from 10.4 to 15.8 MPa (*Table 4*). This improvement is not statistically significant because of the insufficient number of samples treated with CO_2 alone, leading to a high 95% confidence interval. However, it can be reasonably assumed that there is an improvement in the mechanical properties of the bone, very likely due to cross-linking of collagen chains, which is also known to diminish the antigenicity of this molecule. An additional extraction step lowers the compressive strength to a value very close to that of native bone. This phenomenon may be attributed to initiation of collagen matrix destruction. As shown in *Figure 7*, there is only one type of organic molecule and the more complex structure of the organic matter evidenced by the sequential losses after CO_2 extraction disappeared after the second extraction step. Our results are in agreement with those of Poumarat and Squire[20], who found for human and bovine trabecular bone respectively, 8.52 ± 4.24 and 8.78 ± 5.21 MPa.

CONCLUSIONS

The application of supercritical fluid technology to bone tissue appears to be a very promising technique for bone processing. It is the ideal first step of any cascade of bone tissue treatment, since it almost completely eliminates the lipid fraction of medullar tissues without the need for a toxic solvent such as the chlorinated ones commonly used for this purpose. The extraction of the lipid fraction of cancellous bone is essential since the bone fats prevent good recolonization of the implants after grafting by two major phenomena: bone pores are not wettable and lipids themselves may produce antigenic responses.

After the extraction is complete, CO_2 is entirely eliminated just by opening the device to atmospheric pressure, leaving no toxic residue of any kind. Moreover, additional extraction or processing steps are favoured since the microporosity of bone tissue becomes much more accessible. Another advantage resides in the mild operating conditions, which may prevent any damage to the proteinaceous components, especially the bone morphogenetic proteins.

This technique can be applied to human bone tissue in bone banks. Associated with a thorough sterilization step it may bring additional security and efficiency to the orthopaedic surgeon, since osteointegration should be enhanced by careful cleaning of bone porosity.

Obviously, the validity of this technique is dependent on an exhaustive *in vitro*, *in vivo* and clinical evaluation. These studies, which are beyond the scope of this paper, have been performed with very good results, and a commercial xenograft product under the brand name OXBONE® using this technology has recently been launched. Rapid development of the use of supercritical CO_2 for delipidation of bone tissue is expected in the near future.

Table 4 Mechanical properties

	Compressive strength ± confidence interval (MPa)
Native bone	10.42 ± 2.42
Bone after CO_2 extraction	15.83 ± 7.12
After additional H_2O_2 extraction	10.65 ± 2.43
After additional enzymatic extraction	11.54 ± 2.8

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