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INSTRUMENTAL MULTIPARAMETRIC STUDY OF THE MATURING OF THERAPEUTIC MUDS OF SOME ITALIAN SPAS

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ABSTRACT

The problem of the maturing of thermal mud has long been a subject of discussion by many workers, without any objective solution being found.

The process consists of the interaction between a fluid component (mineral water) and a solid component (virgin mud). Maturing is an extremely complex phenomenon which includes not only processes peculiar to the interaction between these components but also a wide range of biochemical processes related to the growth of algae and the micro-organisms characteristic of thermal mud. These processes are influenced by the ecosystem in which maturing takes place.

Maturing involves a complex set of modifications which can be summed up as: (1) modifications related to the virgin mud, (2) appearance of new chemical species or an increase in those already present, and (3) disappearance or decrease in chemical substances already present.

In the present paper, maturing has been investigated using thermal analysis, reflectance spectroscopy, X-ray diffraction, gas, liquid and ionic chromatography, atomic absorption and nuclear magnetic resonance.

Families of molecules and physico-chemical parameters have been analysed to evaluate modifications as a function of time, with the aim of identifying any trends in such modifications which might lead to equilibrium conditions which could be considered indicative of maturing.

INTRODUCTION

Thermal mud is a hyperthermal or hyperthermalized mud produced by the primary or secondary mixing of a solid component with mineral water and, after suitable preparation, is used as a poultice in medical practice.

Thermal mud is therefore characterized by a liquid component, represented by mineral water; a solid component, normally clay, known as virgin mud; and the mixing of the two components, which (after an unspecified

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period of time) leads to the production of the therapeutic mud through the process of maturing.

The process of maturing has been described by numerous authors [1–15] and is known to occur through interaction of the liquid and solid components. It is an extremely complex process which is not limited to a series of specific physico-chemical reactions between the mineral water and the virgin mud, but also involves a number of biological and biochemical processes related to the growth of micro-organisms and algae. These processes are specific to the mud used and are dependent on the habitat determined by the chemical, physical, and physico-chemical characteristics of both the mineral water and the virgin mud. Moreover, as maturing takes place in open tanks, it is also dependent on the biological characteristics of the ecosystem.

Therefore, during mud maturing extremely complex changes are likely to occur in the overall system. These changes can be of three main types.

(1) Changes in the physico-chemical parameters of the clay from which the virgin mud is obtained.

(2) Appearance of new chemical species or the increase of those already contained in the virgin mud, as a result of biological activity.

(3) Disappearance or reduction of the chemical species already present in the virgin mud, as a result of the chemical, physico-chemical or physical action of the mineral water or of biological activity.

Evaluation of the degree of maturity of a mud, hitherto left to the judgement of the practitioner, can be experimentally based on monitoring of the processes described above and on evaluation of whether, how, and over what period of time the maturing process reaches a stationary state (or rather a state of equilibrium). This is possible because the processes involved are always dynamic and the various chemical, physico-chemical and physical characteristics of the mud matrix are either constant or fluctuate over a very narrow range.

Such evaluation, which is also the only type which is experimentally satisfactory, can be performed either by using analytical data to determine whether several parameters have reached a constant value, or by analysing the trends of other parameters and evaluating whether they show an asymptotic tendency. In the latter case, in view of the fact that biological processes are in progress, it may be acknowledged that a condition has been reached which will only be moderately affected even by long periods of time. It is thus possible to accept the beginning of the asymptotic trend as the process cut-off point.

The analysis of individual chemical species which, taken separately, can give only little indication of the development of maturing, and the analysis of all the components of the mud separately (and moreover at different times), would not only be an extremely complex, costly and lengthy process but would also not be repeatable for different spas.

The underlying philosophy of the research has therefore been to study patterns of instrumental observations for families of chemical species and, wherever possible, for highly representative single molecules, or else to study physical parameters indicative of the overall evolution of maturing.

The research has thus followed two main directions: (i) the study of changes in the parameters of the clay matrix, and more specifically, to the species comprising the clay itself, and (ii) the study of the changes in the parameters of the chemical species which coexist with the clay and which are related to the biological processes involved in maturing.

After establishing the type of approach it was developed by sampling at the following stages.

(1) Zero time: virgin mud and mineral water at the source.

(2) 1 day after mixing virgin mud with the mineral water in a normal tank set aside for research purposes.

(3) 3 months after mixing.

(4) 6 months after mixing.

(5) 9 months after mixing (when necessary).

(6) 'Mature' mud normally used for therapeutic purposes and obtained from the same batch of virgin mud.

EXPERIMENTAL

Apparatus

The apparatus used was as follows: a Perkin–Elmer model TGS-2 thermogravimetric system and Perkin–Elmer differential heat analyser equipped with a Data Station; a Beckman DK-2A spectroreflectometer; and X-ray equipment; gas chromatographs (Carlo Erba Fractovap 3900 with SE54 capillary column and FID detector; Dani 3600 for sulphurated compounds Carbopack C + 5% FFAP detector SS, and for chlorides column GP 1.5% SP2250/1.95% SP2401 Supelco Part 100/120 detector ECD); a Perkin–Elmer series 3 liquid chromatograph equipped with LC75 spectrophotometric detector, Spectrofluorescence LS-3, Electrochemical BAS LC-4; an NMR Jeol 270 with ^{13}C, proton and phosphorus nuclei; a Perkin–Elmer model 5000 atomic absorption spectrophotometer equipped with a graphite oven and BC-3 and BC-4 burners, and a MHS-10 system for mercury and iodine determination; a Dionex HPIC (high performance ion chromatography) apparatus equipped with fiber suppressor and chrom/cord ion detector; and an AMEL model 341 apparatus for detecting spring variables.

All the reagents used were from Merck or Carlo Erba, and were of analytical grade.

Analysis of spring water

The dissolved oxygen, conductivity, pH and temperature were measured directly at the spring using an AMEL 341 instrument. The anion and cation analyses were carried out according to the method described in an earlier paper [16].

Preparation of samples

The virgin mud and all subsequent muds were treated before analysis to obtain comparable samples. In particular, the muds corresponding to stages (2)–(5) were placed on a glass filtering partition and left for 24 h to allow the absorbed water to percolate. The samples thus obtained were placed in a ventilated thermostat at 40 °C for 24 h to remove moisture; virgin mud was also subjected to this last treatment.

Study of the changes in the clay species

The samples prepared as described above were subjected to X-ray analysis, thermogravimetry (TG) and differential thermal analysis (DTA), as well

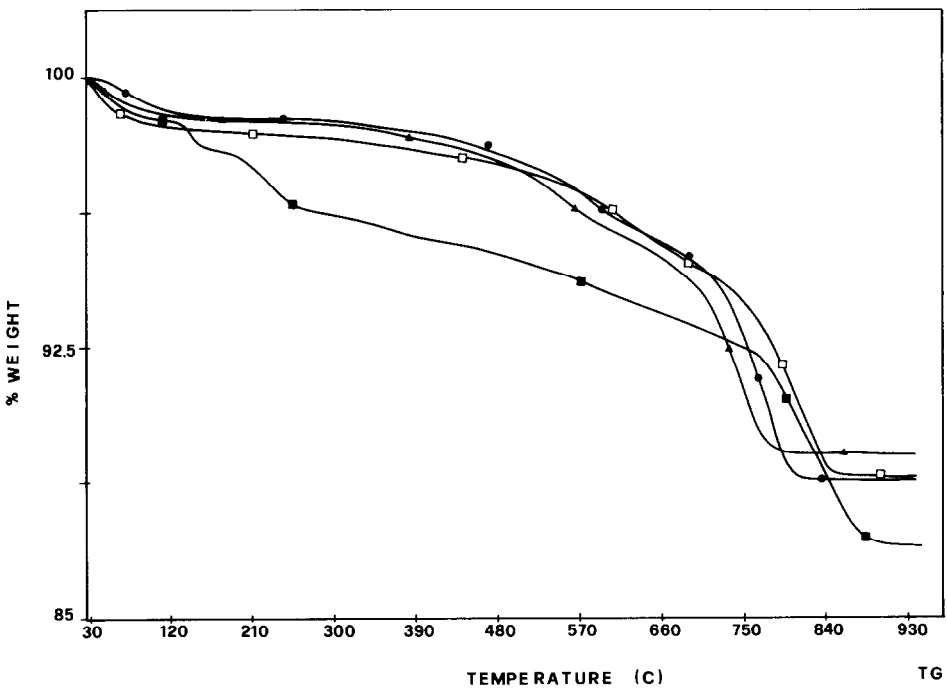

Fig. 1. Thermogravimetric curves of Luigiane Spa mud samples. ●, Virgin mud; ▲, 1-day-old sample; □, 3-month-old sample; ■, 6-month-old sample. Atmosphere, air; heating rate, 10 °C min^{-1}.

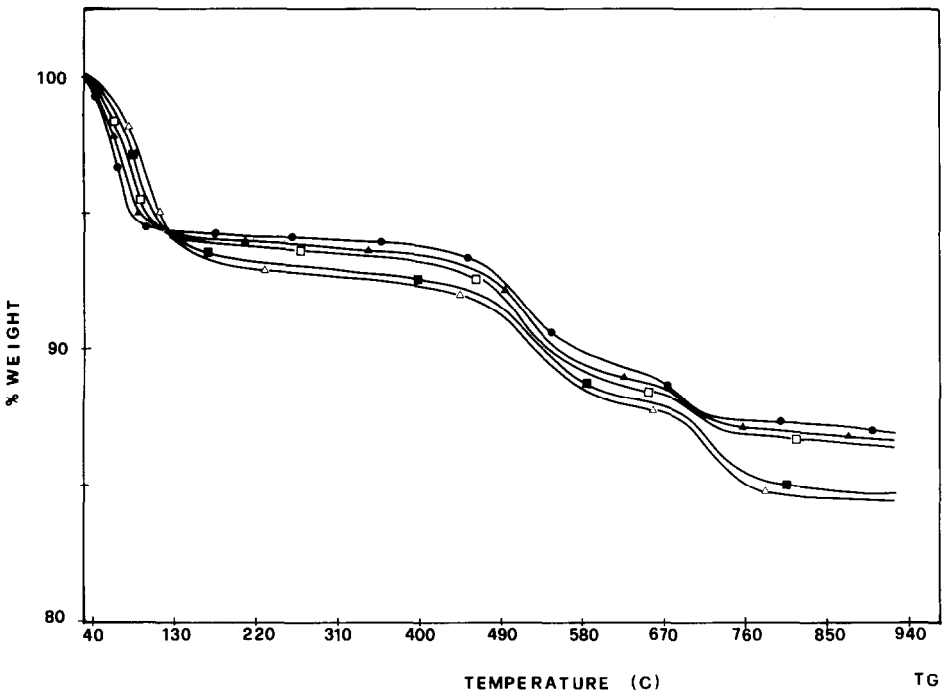

Fig. 2. Thermogravimetric curves of Boario Spa mud samples. ●, Virgin mud; ▲, 1-day-old sample; □, 3-month-old sample; ■, 6-month-old sample; △, 9-month-old sample. Atmosphere, air; heating rate, 10 °C min^{-1}.

as to reflectance spectrophotometry, in order to evaluate possible modifications in the species comprising the clay and in the hydration conditions of the latter.

As well as the above data, several of these techniques also allowed information to be obtained on the biologically related chemical species.

Thermal analysis

The TG curves of virgin mud samples and those corresponding to stages (2)–(5) for three different muds, from the Terme Luigiane, Terme di Boario and Terme di Tivoli spas, are shown in Figs. 1–3, respectively.

The water–matrix interaction increases with time and a change seems to occur in both the percentage of the bonded water, which displays a continual increase with time, and in the type of water present.

The TG curves for the samples of 'mature' muds commonly used for therapeutic purposes are shown in Fig. 4. Also, in this case the water is seen to be given up in the same way as in the corresponding stage (4) or (5) samples, the process temperatures of which coincide with those of samples

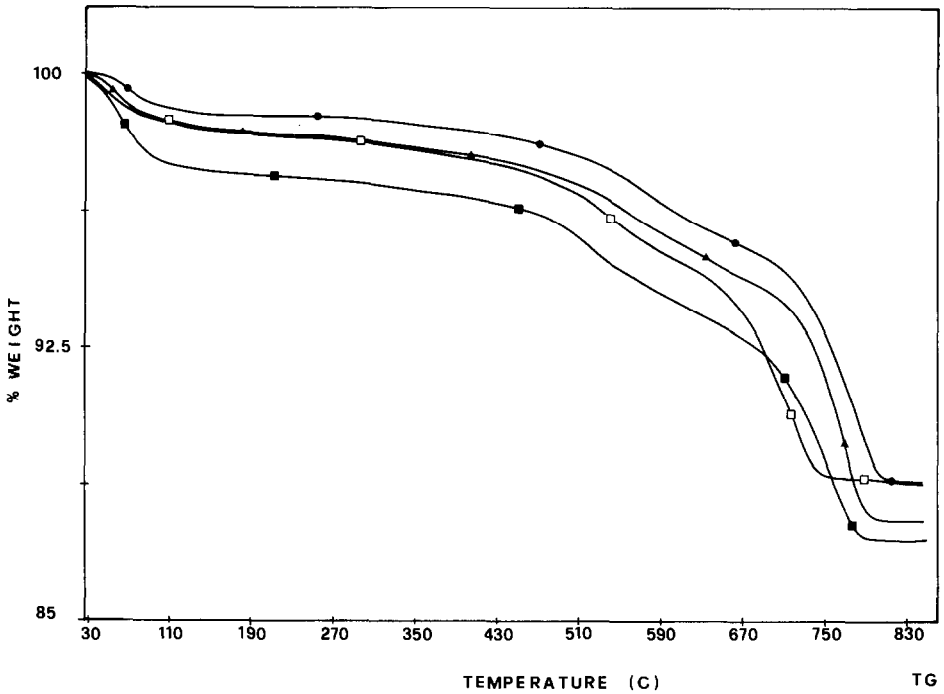

Fig. 3. Thermogravimetric curves of Tivoli Spa mud samples. ●, Virgin mud; ▲, 1-day-old sample; □, 3-month-old sample; ■, 6-month-old sample. Atmosphere, air; heating rate, 10 °C min^{-1}.

from stages (4) or (5). Furthermore, the percentage losses are of the same order of magnitude as those in stages (4) or (5).

The DTA curves are shown in Figs. 5–7. The DTA curves for the corresponding 'mature' muds are shown in Fig. 8, and are seen to be of the same type as those of stage (4) or (5).

Analysis of successive portions of the TG curves reveals the presence of different processes which are very close together. These are very hard to distinguish except when a step characteristic of a particular species occurs, such as the decomposition of calcite. The shape of the curves is a function of both the thermal processes related to the clay components and the oxidative destruction of the organic substances, which takes place at a temperature of 250–650 °C and represents the sum of the thermal processes for the various chemical species of organic origin present in the mud.

The superimposition of these decomposition steps gives rise to a curve which decreases evenly with decreasing temperature throughout the entire thermal process.

Included in this range are of course several processes which involve the minerals contained in the clay. The characteristic steps of these processes are incorporated in the overall decomposition and are therefore unresolved.

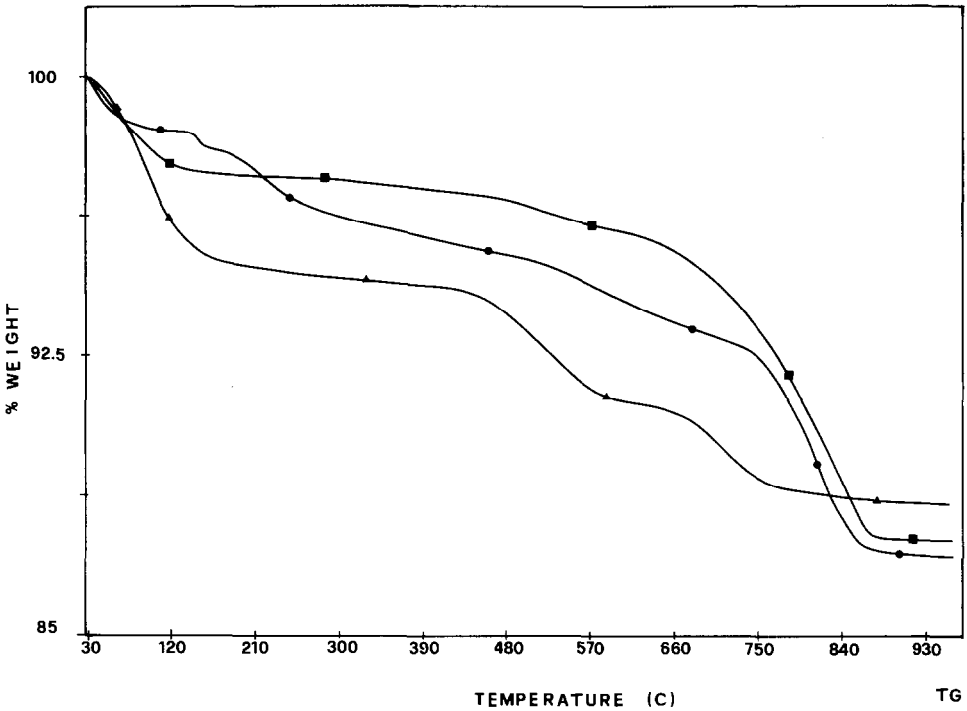

Fig. 4. Thermogravimetric curves of 'mature' mud samples. ●, Luigiane Spa; ▲, Boario Spa; ■, Tivoli Spa. Atmosphere, air; heating rate, 10 °C min^{-1}.

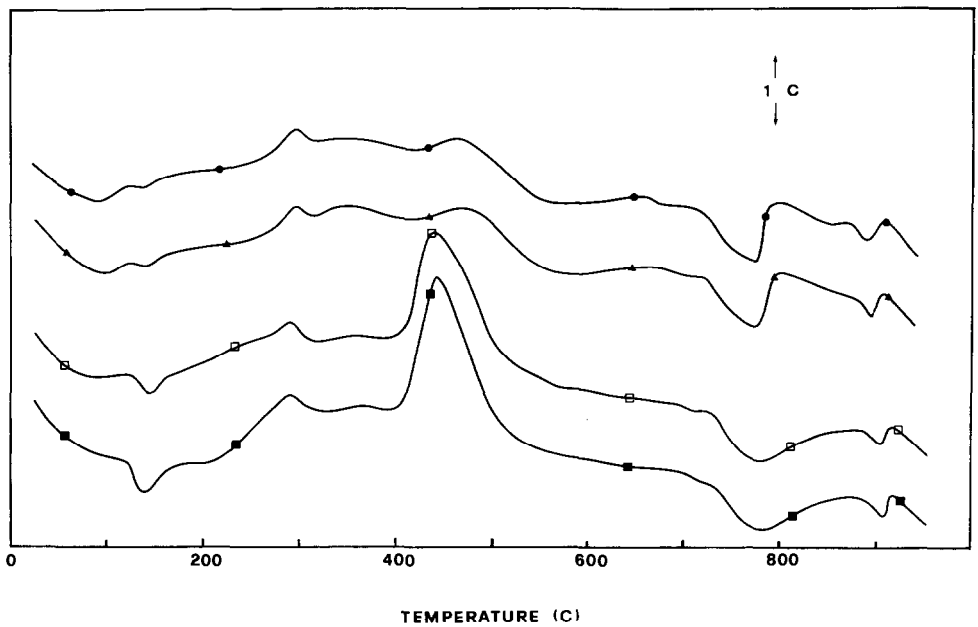

Fig. 5. DTA curves of Luigiane Spa mud samples. ●, Virgin mud; ▲, 1-day-old sample; □, 3-month-old sample; ■, 6-month-old sample. Atmosphere, air; heating rate, 10 °C min^{-1}.

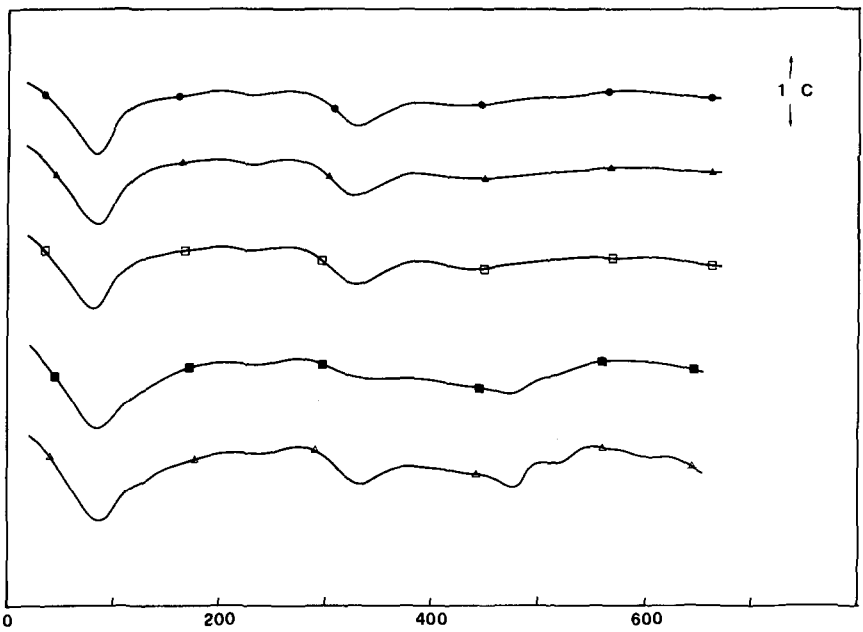

Fig. 6. DTA curves of Boario Spa mud samples. ●, Virgin mud; ▲, 1-day-old sample; □, 3-month-old sample; ■, 6-month-old sample; △, 9-month-old sample. Atmosphere, air; heating rate, 10 °C min^{-1}.

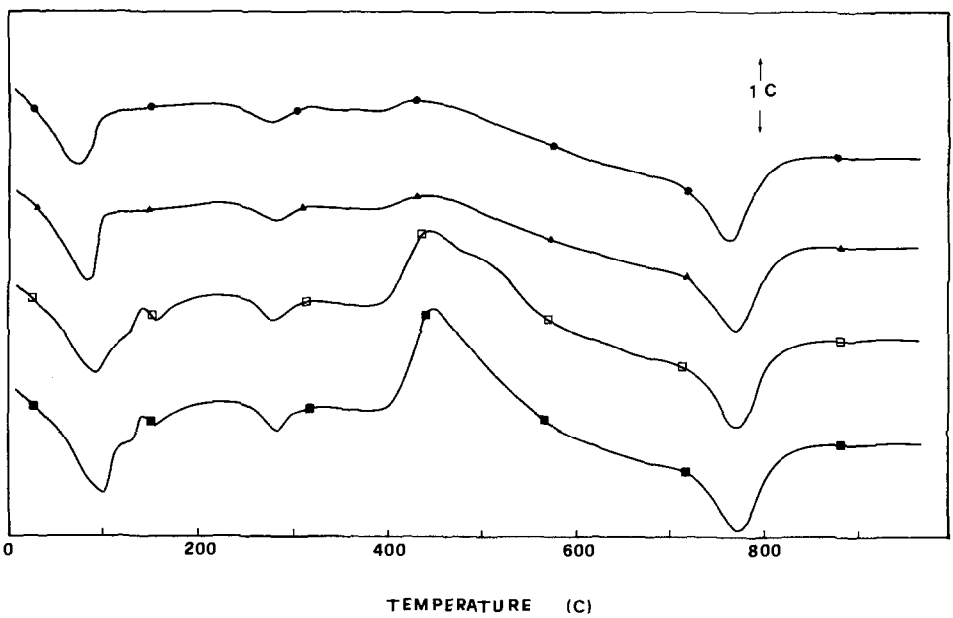

Fig. 7. DTA curves of Tivoli Spa mud samples. ●, Virgin mud; ▲, 1-day-old sample; □, 3-month-old sample; ■, 6-month-old sample. Atmosphere, air; heating rate, 10 °C min^{-1}.

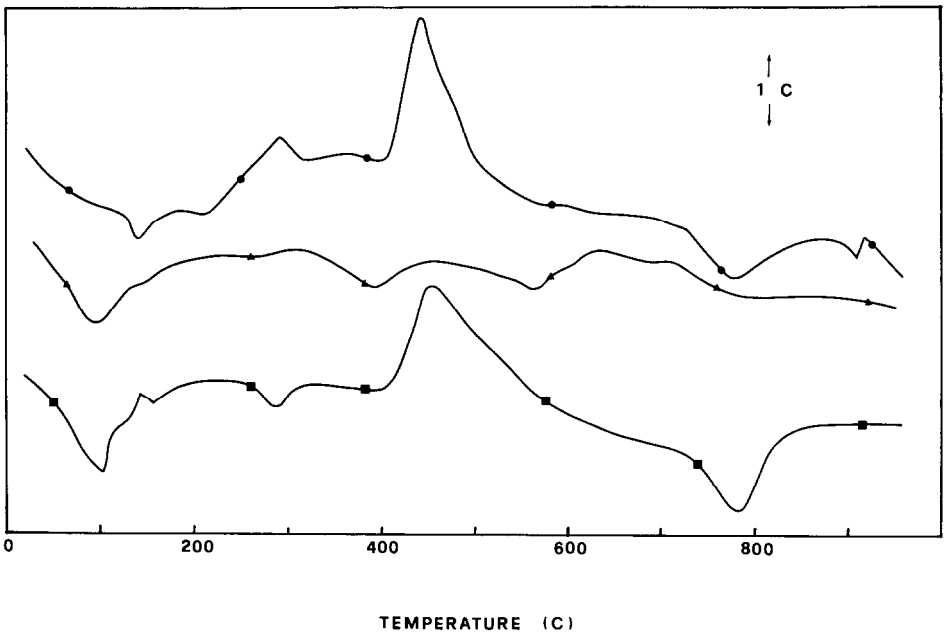

Fig. 8. DTA curves of 'mature' mud samples. ●, Luigiane Spa; ▲, Boario Spa; ■, Tivoli Spa. Atmosphere, air; heating rate, 10 °C min^{-1}.

Despite this lack of resolution, the phenomenological complexity makes the TG and DTA curves extremely characteristic. Thus a pattern which is specific to the system under investigation is obtained, and when the curve for the 'mature' muds is examined and found to be similar to those of stages (4) or (5), it may be concluded, with a high degree of approximation, that the two systems are chemically coherent.

The DTA curves, which have a higher resolution than the TG curves, have peaks which can be ascribed to peculiar phenomena, or to the superimposition of phenomena related to minerals such as illite, montmorillonite, chlorite, calcite and quartz.

The most interesting feature is the considerable increase in the area of the peak with a maximum centered around 440 °C and corresponding to the oxidative breakdown of the organic substances. This indicates that the transition from virgin mud to 6- or 9-month-old mud is accompanied by a considerable increase in the amounts of organic substances.

At the same time, the sharp increase in the width of the thermal demolition band indicates a high degree of complexity of the chemical species present.

The fact that 'mature' or therapeutic muds have DTA curves very similar to those of the corresponding samples from stages (4) or (5) is clearly shown by comparison of Fig. 8 with Figs. 5–7.

X-ray analysis

Both high- and low-angle X-ray analysis of the samples revealed the presence of peaks ascribable to the presence of minerals such as illite,

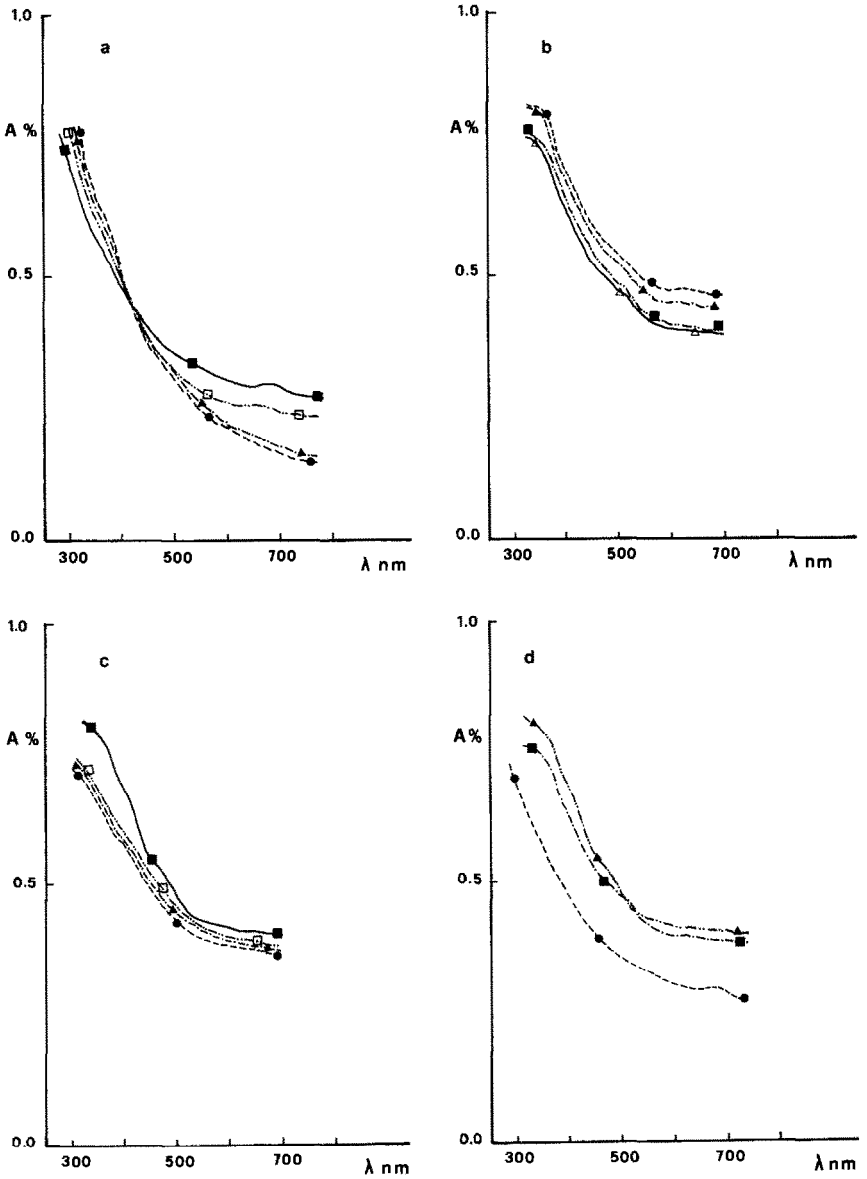

Fig. 9. Reflectance spectra of mud samples. ●, Virgin mud; ▲, 1-day-old sample; □, 3-month-old sample; ■, 6-month-old sample; △, 9-month-old sample. a, Luigiane Spa; b, Boario Spa; c, Tivoli Spa; d, 'mature' mud from: ●, Luigiane Spa; ▲, Boario Spa; ■, Tivoli Spa.

chlorite, montmorillonite, quartz, calcite and feldspar. As an example, for the Terme Luigiane mud, the characteristic peaks for calcite and quartz tend to decrease with time. If this situation is compared with that shown by TG, it can be seen that the decrease in calcite is compressed in time.

The behaviour of the curves for stage (4) or, when necessary, (5) is very similar to that of the sample of mature therapeutical mud. Although this technique is quantitatively affected by several operating conditions, for this reason selected to be as close as possible, the two samples are nevertheless found to be extremely similar.

Reflectance spectroscopy

The next analytical stage consisted in applying diffuse reflectance spectroscopy for the purpose of plotting the overall spectroscopic characteristics of the sample.

The results of this analysis indicate the cumulative evolution of maturing in that the reflectance spectra obtained represent the total of the spectroscopic characteristics of the clay and of the changes in the boundary systems as a result of the chemical, biochemical and biological processes involved in maturing.

The reflectance spectra for samples from the three spas studied are shown in Fig. 9.

Comparing the spectra of the final samples of the muds from these spas with the spectra of the corresponding mature muds (therapeutic muds), it can be seen that they are superimposable.

STUDY OF THE CHANGES IN THE BOUNDARY CHEMICAL SPECIES BY HIGH-RESOLUTION LIQUID CHROMATOGRAPHY (HPLC)

Pretreatment of the sample

Mud samples of 5 g were prepared as described in the preceding analyses and Soxhlet extracted using 50 ml of $CHCl_3$–CH_3OH (1 : 1) for 24 h. The solution thus obtained was reduced to dryness in a rotating evaporator and the residue taken up with 15 ml of methanol–water (85 : 15) mixture. It was then passed through Sep-pak 18 cartridges which had been previously activated by passing 5 ml of methanol and 5 ml of water through them. During this stage any phospholipids present remained in the head of the column and were recovered later. The other substances contained in the mixture were eluted with the mobile methanol–water (85 : 15) phase. The eluate thus obtained was further purified using a column packed with carbon black. After percolation and washing with 2 ml of methanol, the column was eluted with 9 ml of a chloroform–methanol (9 : 1) mixture. The

eluate thus obtained was finally reduced to dryness under in a nitrogen atmosphere and taken up with 100 μl of methanol, and 20 μl were then injected into the instrument.

HPLC analysis

To obtain a differential set of plots which could be related to the different families of chemical compounds, it was decided to use the detector system as

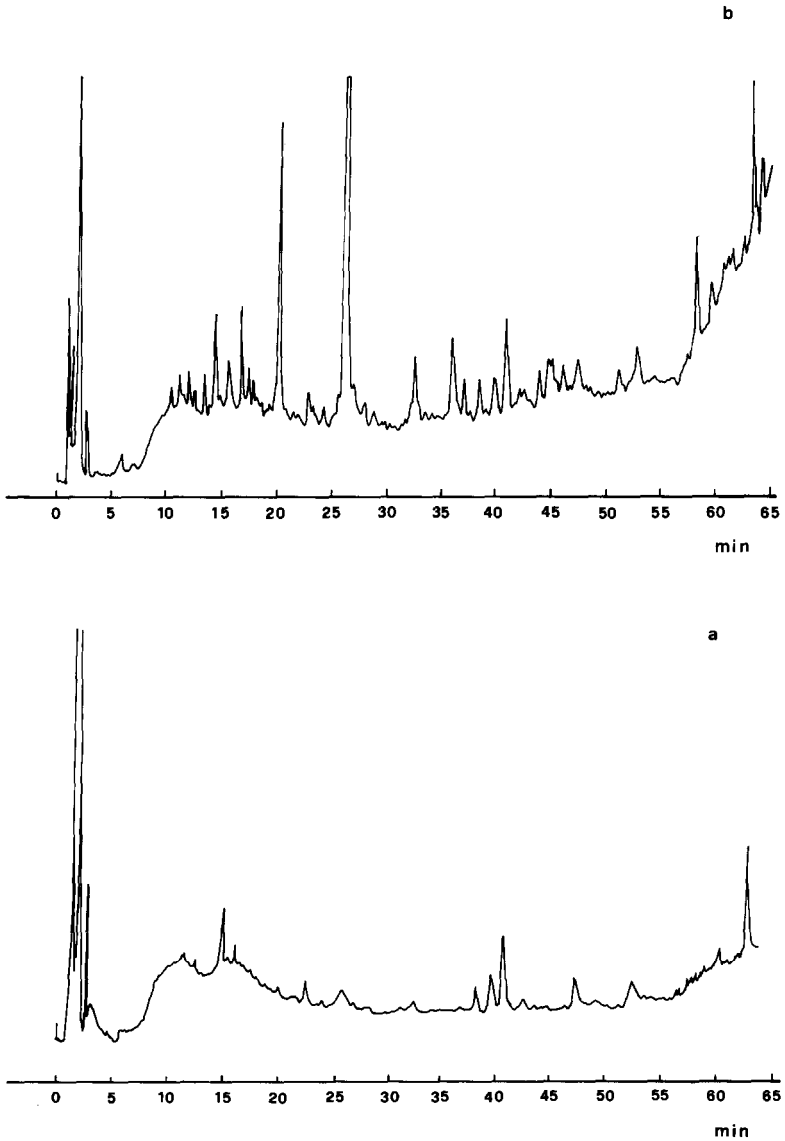

Fig. 10. High-resolution liquid chromatography; spectrophotometric detector: 254 nm. a, Tivoli Spa virgin mud; b, Tivoli Spa 6-month-old sample.

discriminant. Spectrophotometric, spectrofluorescent and electrochemical detectors were therefore used.

The spectrophotometric detector operated at a wavelength of 254 nm, whereas the spectrofluorescent detector was set at 295 nm for excitation and at 405 nm for emission.

The BAS LC-4 electrochemical detector was of the amperometric type with a TL-5 thin-layer vitreous carbon cell and an operating potential of 0.90 V with respect to a silver/silver chloride reference electrode.

Using the UV detector produced very complex chromatograms, in view of the wide range of substances that can be detected by this system and that could be related to aromatic compounds and to all those compounds that, because of the presence of double bonds, can be absorbed at this wavelength. Although, analytically, the plot is difficult to use, owing to its high degree of complexity, from the graphic point of view its complexity makes it useful for revealing relationships with the degree of maturity. The discussion will be restricted to the mud of the Terme di Tivoli.

The plots for the zero time curve (a) and 6-month curve (b) are shown in Fig. 10. Considerable changes are seen to have occurred in the chromatograph pattern, with a number of species disappearing from the virgin mud and new species appearing in the 6-month-old mud, and there are often great changes in the concentration of species present from the outset.

The plot for the 'mature' mud sample is shown in Fig. 11. Comparison with curves a and b in Fig. 10 shows that, except for some minor differences, the shape closely resembles that of curve b in Fig. 10, i.e. that of the 6-month-old sample.

The use of a fluorescence detector, whose response is related to the presence of chemical species of the alcohol, phenol and sterol type (in general to the presence of all systems that, after excitation, can fluoresce), produces plots that are less complex and more selective than those obtained using a UV detector. Comparison of the two curves corresponding to stages (1) and (4) shows that the plot for stage (4) displays a larger number of species with low retention times (i.e. more polar species), is much poorer in species with medium retention times (i.e. with medium polarity) and loses very little in the case of substances with high retention times (i.e. species with low polarity).

The chromatogram for the 'mature' mud consists of a graph which closely resembles that of the stage (4) sample.

Lastly, an electrochemical detector was used to increase selectivity and, at the same time, to obtain instrumental signals which could be related to the chemical species and which differ, at least in part, from those detectable using UV and fluorescent detectors. This type of detector allows the detection of oxidizable substances, although at the potential at which the detector is operating, such as amines, amino acids, phenols, catechols, etc. The plots obtained using this detector show that a considerable reduction had oc-

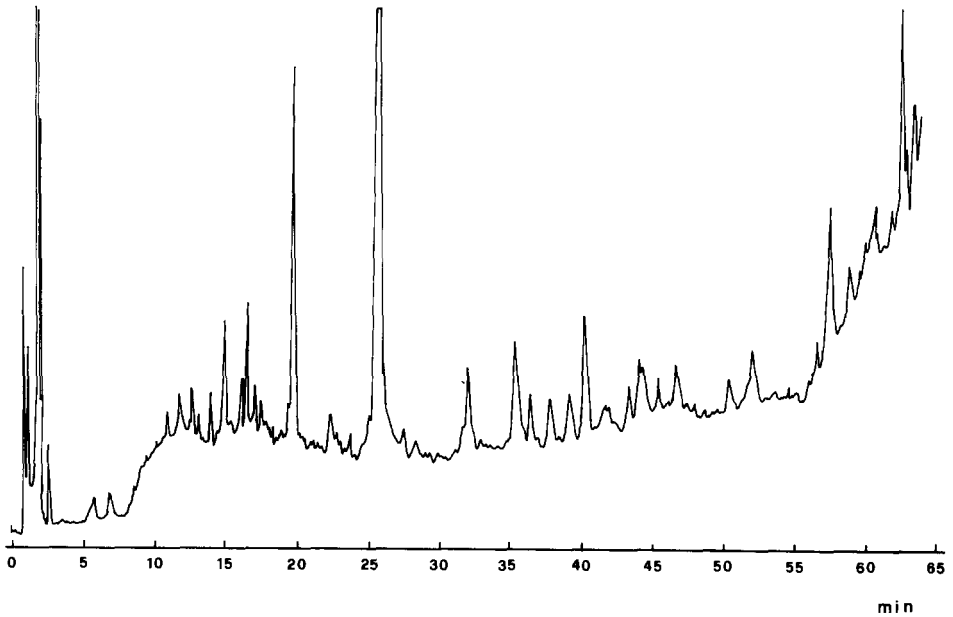

Fig. 11. High-resolution liquid chromatography; spectrophotometric detector: 254 nm. Sample of Tivoli Spa 'mature' mud.

curred in the higher retention time peak, and that the immediately preceding peak has disappeared. A parallel reduction occurs in the overall chemical complexity of the system.

Examination of the curve for the 1-day-old, stage (2), sample, shows that this sample is already radically different from virgin mud, and its plot refers to a situation which is tending rapidly towards that of the 6-month-old mud, and which is already very similar to the 3-month-old stage. This implies that the substances detected in the first sample, which were tending to disappear very rapidly, are highly soluble oxidizable substances which are rapidly washed out, or are substances which disappear through rapid kinetic reactions with the components of the mineral water.

The curve for 'mature' mud closely resembles the stage (4) curve.

It is also important to note that the 3-month curves obtained using the different detectors tend to resemble the 6-month curves, i.e. several peaks have already taken on the shape of those for stage (4), and others are approaching this situation. The mud therefore tends towards a stationary state.

GAS CHROMATOGRAPHY (GC)

Pretreatment

Mud samples of 5 g pretreated by percolation and ventilation as described above were Soxhlet extracted with 50 ml of a chloroform–methanol (1 : 1)

mixture. The solution thus obtained was reduced in a current of nitrogen to a volume of 2 ml and subsequently chromatographed on a 60 F 254 20 × 20 cm silica gel preparation slide, 0.25 cm thick, using a benzene–hexane (4 : 6) mixture as eluent. This method was used to separate aliphatic hydrocarbons from polycyclic aromatic compounds with $R_f = 0.85 + 1$ and $R_f = 0.6 + 0.85$, respectively.

The bands thus obtained were removed from the slide, eluted with suitable solvents and quantitatively extracted, and the solutions obtained were subjected to GC analysis.

Attention was focused on several families of compounds, in particular hydrocarbons, chlorinated compounds, sulphurated compounds, and polycyclic compounds. In view of their well-known implications in health problems in general, and in dermatology in particular, it was deemed of interest to determine their presence, if any.

The chromatograph curves for the analysis of polycyclic hydrocarbons show that these compounds are absent both in the sample of virgin mud and in the 6-month-old mud.

Next to be considered were hydrocarbons, taking into account the fact that Ciferri [1] had indicated the presence of paraffins in humus, soil and peloids. Furthermore, according to Curri et al. [5], hydrocarbons must not be underestimated as factors in peloid 'maturing' and perhaps in certain physico-chemical repercussions when they come in contact with the skin.

Therefore, GC analysis was carried out on the extracts corresponding to the bands on the silica gel slide which indicated the presence of hydrocarbons.

The chromatograms for zero time and 6 months show that there is a sharp increase in several peaks and a large reduction in others, which indicates a highly dynamic situation.

Analysis of the 3-month sample points to clearcut modifications with respect to zero time, and a pronounced tendency towards the situation reflected in the 6-month old sample.

The curve for the 'mature' mud is very similar to that for the 6-month-old sample.

Analysis of the chlorinated compounds, whose dermatological reactivity is well known, was carried out directly on the extract.

Extensive changes are visible, particularly in the compound with higher retention times. The curve, for stage (3) (3 months), shows that the situation relative to this family of molecules has almost reached a stationary state. This is confirmed by comparison between the curve for the 'mature' mud and that for the 6-month-old mud.

Lastly, any sulphurated compounds present were analysed directly on the extract using a sulphur detector. It was found that no sulphurated compounds were present in samples for stages (1) and (2), whereas two characteristic peaks were visible in samples for stages (3) and (4). In accordance

with the findings of Rampazzo and Gonzato [17] and on the strength of the position of the band on the chromatographic slide, these peaks were ascribed to liposoluble sulphurated compounds related to the presence of algae.

The same peaks, with characteristics similar to those of the stage (4), 6-month-old mud, were found in the 'mature' mud. It is noteworthy in this case that the two peaks have a slightly larger area. This can be accounted for by the fact that, as Rampazzo has pointed out, ageing of the algae leads to enrichment in sulpholipids.

Phospholipids

After washing the Sep-pak 18 column with a water–methanol (1 : 1) mixture, the phospholipids which, as mentioned above, had remained in the head of the column, were eluted with 10 ml of pure methanol.

The eluate was then divided into two aliquots, one of which was dried and then taken up with deuterochloroform $CDCl_3$ and subjected to NMR analysis. The other aliquot was used for in toto phospholipid analysis. In toto phospholipid dosage was achieved using a direct enzymatic method based on the use of phospholipase-D [18]. This method is highly specific and sensitive. The analyses performed at stages (1) and (2) gave negative results, while those carried out at stage (3) indicated the presence of phospholipids in amounts up to 0.000 035% of the weight of the dry mud sample, corresponding to 0.17 mg.

The analyses performed at stage (4) indicated a quantity of phospholipids equal to 0.000 09% of the weight of dry mud, corresponding to 0.45 mg.

Lastly, the analyses at stage (5) ('mature' mud) indicated the presence of phospholipids in quantities equal to 0.000 11% of the weight of the dry mud sample, corresponding to 0.55 mg.

Nuclear magnetic resonance was used to evaluate the complexity of the phospholipid mixtures, using phosphorus, proton and ^{13}C nuclei.

These tests revealed the presence of phospholipids only at stages (3), (4) and (5).

At stage (3) the signals were very weak compared with those at stages (4) and (5). This was in agreement with enzymatic analysis. The signals were comparable, however, at stages (4) and (5).

The signals obtained were ascribed to the presence mostly of phosphatidylcholine, and of traces of sphingomyelin and phosphatidylethanolamine.

CONCLUSIONS

The data set for the samples analysed, with regard to both the clay matrix and the boundary chemical species, reveals a tendency to move towards a stationary state or at least towards an asymptotic phase which, depending

on the virgin mud and mineral water types, occurs between 6 and 9 months after the beginning of maturing.

Future investigation should involve the analysis of single molecules, or at least of molecules that are highly representative of and significant for each mud and to evaluate their pharmacological activity.

At the same time it would be interesting to evaluate the mud's action on the skin, by investigations of the cutaneous modifications induced by mud therapy.

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