BIOACTIVE COMPOUNDS EXTRACTION FROM POMACE OF FOUR APPLE VARIETIES

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Abstract: This paper describes a comparative study of extraction methods such as maceration, pressurized liquid extraction, ultrasound assisted extraction or microwave assisted extraction with different solvents of bioactive compounds from pomaces of four different apple varieties. In order to evaluate their chemical composition the obtained extracts were analysed by High Performance Liquid Chromatography – UV detection – Evaporative Light Scattering Detection (HPLC-UV-ELSD). The study revealed that, while extraction solvent influences the recovery of different compounds, the extraction techniques do not induce selectivity since any major differences of the extracts fingerprints and of the relative proportion of compounds were observed.

Keywords: apple pomace, extraction, maceration, pressurized liquid, ultrasound, microwave, bioactive compounds

1. INTRODUCTION

Nowadays there is an increasing interest for the recovery of bioactive compounds from natural sources due to their beneficial effects on the human health. These compounds can present anti-inflammatory, anti-carcinogenic or antioxidants properties. They can also block the activity of bacterial or viral toxins, inhibit cholesterol adsorption, destroy harmful gastrointestinal bacteria etc. [1].

Produced in important quantities each year, apples are considered as an important source of bioactive compounds. Often these fruits are processed for juice recovery leading to large quantities of by-products (30% of raw material) known as "apple pomace" and represented by fruit pulp, peels, seeds etc. Pomace may still contain different functional components such as polysaccharides, vitamins, fibres, polyphenols or triterpenes (a less studied group of apple compounds).

This work was aimed to evaluate the possibility to develop bioactive compounds from different pomace apple varieties as relevant ingredients that could be incorporated in food, cosmetic or pharmaceutical formulations.

An important step in the qualitative and / or quantitative analysis of apple pomace compounds is the extraction process which insures the separation of these bioactive compounds from the cellular matrix. The efficiency and the selectivity of the extraction process could depend largely on the technique, the solvent, the energy input and

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the agitation that can improve the chemical solubility and efficiency of mass transfer. The traditional techniques (maceration or Soxhlet extraction) are widely used because they are relatively easy to perform and cheap to operate but they usually require long extraction time, high energy consumption, intensive manual procedures and may have low efficiency. These disadvantages can be eliminated by using other methods such as pressurized liquid extraction, ultrasound assisted extraction or microwave assisted extraction.

The extracts obtained from pomace of four apple varieties (Gala, Golden, Granny Smith, Pink Lady) using the above mentioned extraction techniques were analysed by liquid chromatography in order to evaluate the chromatographic profile of the extracts according to the extraction solvent, extraction technique, and apple variety used.

2. MATERIALS AND METHODS

2.1. Extraction methods description

2.1.1. Maceration (M)

Maceration is the most simple solid-liquid extraction method and consists in placing the plant material in contact with the solvent with or without stirring at room or elevated temperature for a given period of time. This technique is based on the solubility of the bioactive compounds in an extraction solvent and it is influenced by a series of factors including type of vegetal matrix, solutes concentration in the sample, solvent nature, extraction duration etc. Maceration begins with the appropriate choice of the extraction solvent. After a step of solvent diffusion inside the plant cells the process continuous with the solubilisation of the bioactive compounds which (once dissolved) will migrate from the vegetal matrix to the surrounding solvent until this is saturated.

2.1.2. Pressurized liquid extraction (PLE)

Pressurized liquid extraction is applicable for the extraction of many compounds from food and agricultural materials such as carotenoids from processed foods [2] or antioxidants from microalgae [3].

This technique uses the same solvents as maceration, but at temperatures above the boiling point of the extraction solvent (50–200°C) and at an increased pressure (100–140 atm) which allows maintaining the solvent in a liquid state at a high temperature. Under these conditions, the solvent has properties favouring the extraction process, such as low viscosity, high diffusion coefficients, and high solvent strength conducting to good kinetics of dissolution processes and favouring desorption of analytes from the plant matrix [4].

Figure 1 illustrates a pressurized liquid extraction system. The solid sample is enclosed in a stainless steel extraction cell that is placed in an oven and filled with an extraction solvent. The heating process generates solvent expansion and thus pressure in the extraction cell. In order to prevent over-pressurization of the cell, a static valve pulses open and close automatically when the cell pressure exceeds the set point. The solvent that escapes during this venting is collected in a vial. A static extraction stage is followed by pumping fresh solvent through the system to rinse the sample and the tubing. All the solvent present in the system is then purged with a compressed gas, generally nitrogen, into the collection vessel [5 - 6].

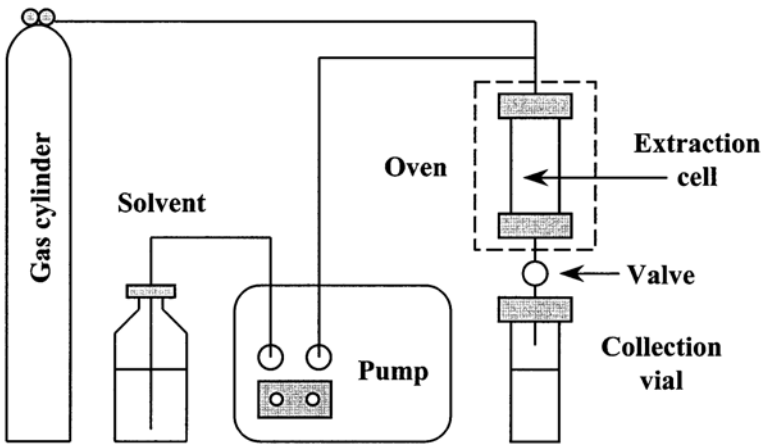

Fig. 1. Schematic representation of pressurized liquid extraction system [7].

2.1.3. Ultrasound assisted extraction (UAE)

Another effective way to extract analytes from different matrices [8 - 11] in short time is by using ultrasound assisted extraction. Ultrasounds are mechanical waves at a frequency above the threshold of human hearing that can provide a better penetration of solvent into plant materials and improve the mass transfer.

The effect of ultrasound on a fluid is due to acoustic and hydrostatic pressure action on the medium. At low intensity, the acoustic pressure induces motion and mixing within the fluid. In the expansion phase of a cycle, at higher intensities, the local pressure falls below the liquid vapour pressure, causing the growth of the bubbles created from existing gas nuclei within the fluid. This increase generates negative transient pressures enhancing bubbles growth and producing new cavities by the tensioning effect on the fluid. When a critical size range is reached the bubbles implode during the compression cycle. This process of compression and rarefaction of the medium particles and the consequent collapse of the bubbles is called "cavitation"[12].

As represented in Figure 2 the cavitation bubbles can be generated close to the plant material surface (a). Then during a compression cycle, this bubble collapse (b) and a microjet directed toward the plant matrix is created (b and c). The local high pressure (1000 atm) and high temperature (5000 K) involved in this process can destroy the plant matrix cell walls and its content can be released into the surrounding medium (d).

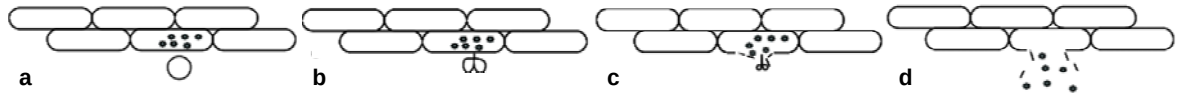

Fig. 2. Cavitation bubble collapse and plant material releasing [13]:
a. cavitation bubble generation; b. bubble collapse; c. microjet creation; d. analytes release.

2.1.4. Microwave assisted extraction (MAE)

Discovered mathematically by Maxwell and experimentally verified by Hertz microwaves are high frequency electromagnetic radiations ranging from 300 MHz to 300 GHz, with wavelengths between l m and l mm [14].

In conventional extraction techniques, when heating, the thermal energy is transferred from exterior to the interior of the plant material through conduction and convection. In microwave assisted extraction the electromagnetic waves penetrate into the sample and their energy is transformed into heat through ionic conduction and dipole rotation. Ionic conduction refers to the movement of ions in a solution under an electromagnetic field. The friction between the solution and the ions generates heat.

The second mechanism (Figure 3) is based on the fact that polar molecules (like water, methanol, ethanol etc.) are in fact dipolar presenting positive and negative ends. In normal state the molecules of a product possessing dielectric properties are in Brownian moving. Under the effect of an electric field the molecules begin to orientate themselves. More the electric field intensity is greater less the thermal agitation is important. When all molecules are oriented a global dipole moment is installed. Under microwave radiation the polarized molecules orient themselves into the field direction, they disorient if the microwave action disappears and orient again in the opposite direction when a microwave radiation is reapplied [15 - 16].

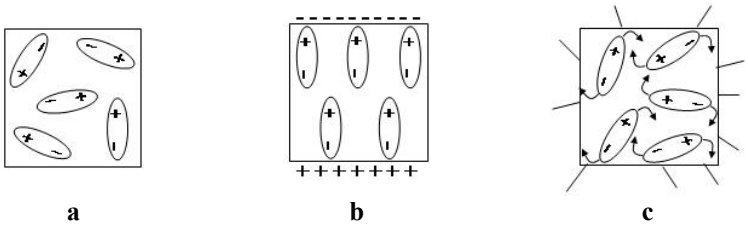

Fig. 3. Dipolar molecules behaviour [17]:
a. thermic movement; b. electric field action; c. microwave action.

The interaction forces between molecules (hydrogen bonds, Van der Waals bonds) that oppose to the free molecules rotation cause internal frictions conducting to heating. Under the microwave action the dipolar molecules align rapidly with the electromagnetic field (4.9×10^9 times per second) and start to vibrate also with

heat releases [18]. This heat conduct to a sudden and important increase of temperature inside the extraction solvent and/or inside the plant material cells causing an internal overpressure and leading finally to their rupture. The cells compounds can, migrate easily to the surrounding solvent which can dissolve them [19]. Among the different analytes obtained using this extraction technique we can cite phenolic compounds [20 - 21], terpenes [22 - 24] or pectin [25 - 26].

2.2. Reagents

Methanol, ethanol and ethyl acetate used for extraction and HPLC analysis were of analytical grade and were purchased from SDS Carlo Erba - France. Formic acid, gallic acid (GA), chlorogenic acid (CA), catechine (CAT), betulinic acid (BA) and oleanolic acid (OA) were purchased from Sigma Aldrich (Saint Quentin Fallavier, France). Ursolic acid (UA), uvaol (UV), rutin (RUT), quercetin (QUE), catechin (CAT), phloridzin (PH) were purchased from Extrasynthèse France.

The water used for extraction, for the HPLC mobile phase or for standards solution preparation was purified by an Elgastat UHQ II system (Elga, Antony, France) from distilled water (resistance < 18 MΩ).

2.3. Plant material

Five different samples of apple pomaces were used in this study. The industrial Granny Smith sample was procured from the French factory "Touraine Jus de Pomme".

Four home-made samples from Gala v. Royal Gala Tenroy, Golden v. Golden, Granny Smith and Pink Lady v. Cripps Pink apple varieties were prepared from quantities of approximately 1 kg of apples cut into slices and pressed in a home pressing machine. The resulting pomaces were immediately packed in plastic bags, frozen at – 80°C for 24 hours and then lyophilized for 72 hours. The obtained dried pomaces were ground to powder and stored at room temperature in closed glass recipients until extraction.

2.4. Extraction procedure

2.4.1. Maceration

1 g of each dried apple pomace was extracted with 20 mL of solvent in a 50 mL closed glass bottle at room temperature during 60 minutes while agitating with a stir bar.

2.4.2. Pressurized liquid extraction

Extraction was performed on an ASE 100 Accelerated Solvent Extractor from Dionex (France). 3 g of dried apple pomace were mixed with the same quantity of Na_2SO_4 as dispersive reagent and packed inside a 34 mL stainless steel extraction cell with a cellulose filter placed at the bottom. 3 consecutive extraction cycles were carried out at 40°C with a static time of 5 min per cycle, 65% flush volume and a purge with nitrogen of 100 s after each extraction. A volume of approximately 60 mL of extract was recovered.

2 4.3. Ultrasound assisted extraction

For the ultrasound assisted extraction 3 g of dried sample and 60 mL of solvent were placed inside a 500 mL Erlenmeyer and sonicated in a rectangular ultrasonic bath for 30 minutes at room temperature.

2.4.4. Microwave assisted extraction

Microwave assisted extraction was carried out using a Milestone MicroSYNTH microwave oven (Sorisole, Italia) with adjustable power settings and irradiation time controlled by ''easyCONTROL'' software. Temperature was followed by an infrared external sensor able to control the exterior reactor temperature.

1 g of dried apple pomace and 20 mL of solvent were introduced in a 50 mL glass reactor vessel and submitted to an extraction in 3 cycles of 30 s using a microwave power set at 1000 W. Between the cycles the reactor was cooled down on ice until the room temperature was achieved.

All the extracts were centrifuged at 5000 rpm for 5 min at 15°C and the supernatant liquid evaporated to dryness in a rotary evaporator under vacuum at 40°C. The quantities of the obtained dried extracts were reported at those of plant material used for extraction in order to calculate the extraction yields. The dried residues were re-dissolved in 5 mL MeOH, centrifuged in the same conditions as the crude extracts, filtered through a 0.45 µm pore size syringe filter, stored at 4°C and submitted to HPLC analysis after dilution to an adequate concentration.

2.5. HPLC analysis

Separation and detection of all the extracts compounds were achieved by HPLC-UV-ELSD analysis in reversed-phase mode on a Varian Pursuit XRs C18 analytical column (150 x 4.6 mm, 5 µm) attached to a RP-C18 safeguard column. A mobile phase, at a flow rate of 1 $mL.min^{-1}$, consisting of water as solvent A and methanol as solvent B both acidified with 0.1% of formic acid was used in a gradient elution program as follows: 20% to 90% B from 0 to 30 min, 90% B from 30 to 45 min. The column was operated at room temperature.

The chromatographic analyses were carried out using a LaChrom Elite HPLC apparatus interfaced with a personal computer utilizing the EZChrome Elite workstation software for control and data collection. The system was equipped with a degasser, an auto-sampler, a Diode Array Detector (DAD) and a quaternary pump and coupled to an Evaporative Light Scattering Detector (ELSD) type Sedex 85 from Sedere – France. A sample volume of 20 µL was injected and the signals were recorded by DAD from 200 to 600 nm and by ELSD using a drift tube temperature of 52°C; a nebulizer gas pressure of 3 bars and a value of the gain fixed at 6.

3. RESULTS AND DISCUSSION

3.1. Comparison between extraction methods

Generally the conventional methods for extracting natural compounds are mainly maceration and Soxhlet extraction [27 - 28]. In spite of their efficiency these processes usually need long time and consume important volumes of solvent. These disadvantages can be avoid by using alternative extraction techniques such as ultrasound assisted extraction, microwave assisted extraction or pressurized solvent extraction. The extracts obtained by these extraction methods were compared to those resulted after traditional maceration. As indicated in Figure 4, the best global extraction yield (55%) was achieved by MAE, similar extraction yields (around 43%) were achieved in the case of maceration (M) and UAE while the PLE technique gave the lowest yield (only 33%).

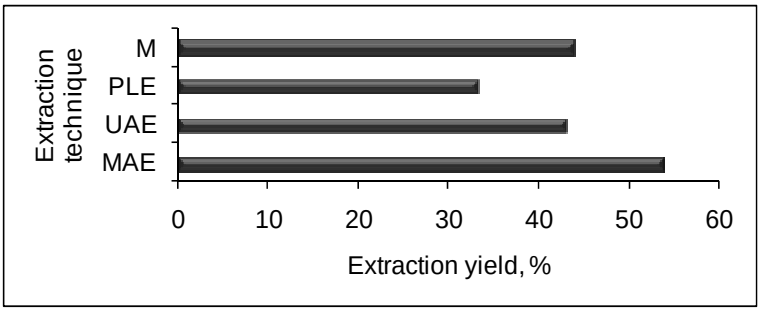

Fig. 4. Comparison of extraction yields for Granny Smith pomace ethanolic extracts obtained by different extraction techniques.

We can note on the ethanol extract HPLC profiles that the extraction techniques do not induce selectivity since no major differences of the extracts fingerprints and of the relative proportion of compounds were observed. Knowing that MAE was the quickest process since only 3 cycles of 30 sec each were carried out and by consequence the less energy consuming it was chosen for further experiments.

3.2. Influence of the extraction solvent

The recovery of bioactive compounds from apple pomaces is influenced by the solubility of these compounds in the solvent used for the extraction process. Furthermore, solvent polarity will play a key role in increasing the solubility. In order to evaluate the influence of the solvent on the extraction process mixtures of 1 g of Granny Smith industrial pomace and 20 mL of different solvents such as water / methanol mixture (10:90), pure ethanol or pure ethyl acetate were submitted to microwave assisted extraction.

We have noted that in the presence of water, a rehydration process of dried apple pomace takes place. This process is accelerated by the increased temperature under microwave irradiation making very difficult the extract recovery. Thus the extraction of bioactive compounds from apple pomace was not conducted with pure water.

As shown in Figure 5, the recovery of bioactive compounds was dependent on the solvent used and its polarity. It can be observed that water / methanol mixture extracts mostly polar compounds (eluted before 20 min) and ethyl acetate is an adequate solvent for the least polar ones (eluted after 15 min). On the other hand, the polarity of ethanol is a good compromise for the extraction of both types of compounds.

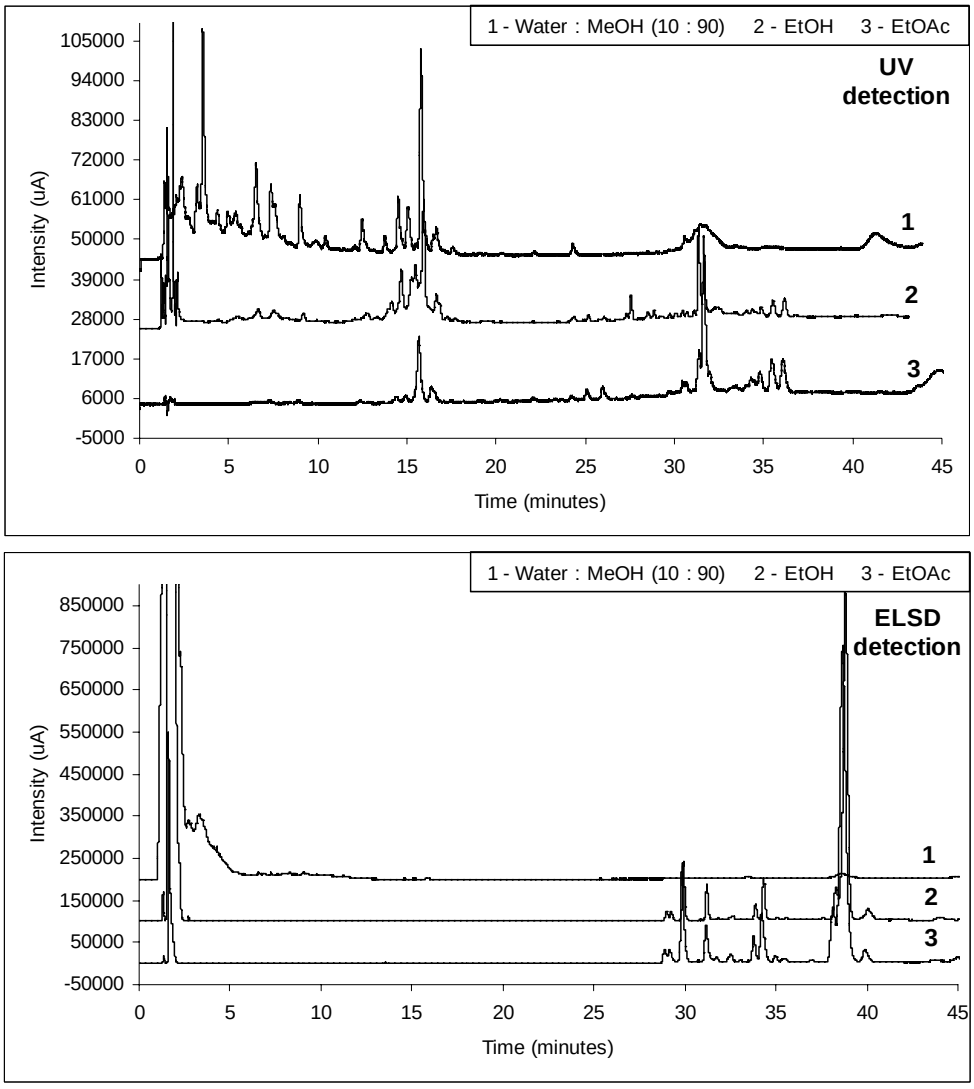

Fig. 5. Comparison of industrial Granny Smith pomace extracts obtained with different solvents under microwave conditions Column: Varian Pursuit XRs C18 (LxΦ = 150x4.6 mm, 5µm) Detection: UV 280 nm; ELSD: drift tube temperature, 52°C; nebulizer gas pressure, 2 bars; gain, 8 Mobile phase: A. H_2O; B. MeOH both with 0.1% HCOOH; Flow-rate: 1 $mL.min^{-1}$ Elution gradient: 0–30 min, 20–90% B; 30–45 min, 90% B.

When comparing the chromatographic fingerprints obtained by the two types of detection it can be seen that the apple pomace samples contain small quantities of compounds with a higher polarity (eluted before 20 min and detected by UV) while the least polar compounds are more abundant.

3.3. Comparison between pomaces from different apple varieties

The chromatographic profile of the ethyl acetate extracts of home-made pomaces is represented in Figure 6. The same compounds were observed for all the pomace samples with different peaks intensity. This conclusion is sustained also by the chromatographic fingerprints of the ethanolic extracts obtained under microwave conditions. Pink Lady seemed to be the richest variety, with higher peak intensity.

An attempt to identify the apple pomace compounds was realized. Different standards solutions were analysed in the same chromatographic conditions as the pomace extracts and their retention time (Table 1) and absorption spectra were compared to those of the extracted compounds. The results indicate the presence of phenolic

compounds such as gallic acid, chlorogenic acid, catechine, rutine or quercetine and of triterpenes like betulinic acid, oleanolic acid or ursolic acid.

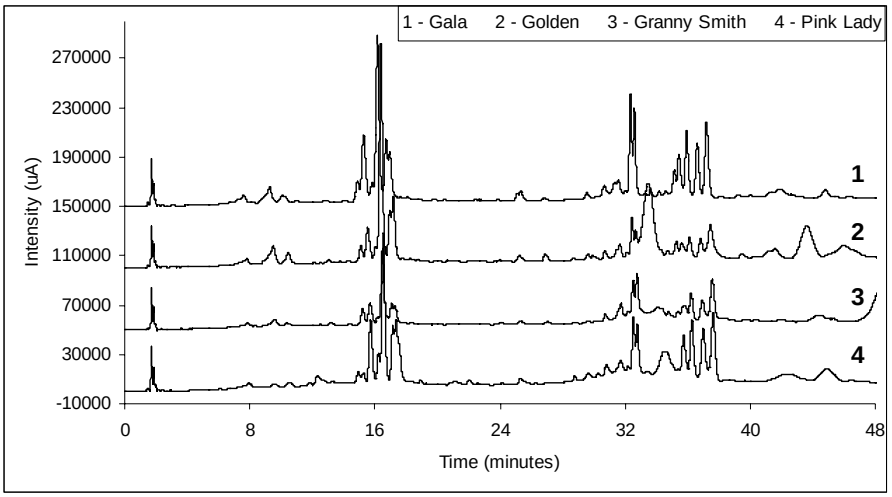

Fig. 6. Comparison of chromatographic profiles of different apple pomace ethyl acetate extracts obtained by maceration Column: Varian XRs C18 ((LxΦ = 150 x 4.6 mm, 5µm); Detection: UV 280 nm Mobile phase: A. H_2O; B. MeOH both with 0.1% HCOOH; Flow-rate: 1 $mL.min^{-1}$ Elution gradient: 0 – 30 min, 20 – 90% B; 30 – 45 min, 90% B.

Table 1. Standards retention time.

Standard	GA	CAT	CA	RUT	PH	QUE	BA	OA	UA
Retention time (min)	3.46	7.14	8.36	16.16	16.83	20.41	38.86	40.10	40.67

4. CONCLUSION

In this study the efficiency of different compounds transfer into various solvents from dried apple pomaces by alternative extraction techniques were compared. The MAE method employed with ethanol as solvent was found to be the most appropriate combination for the extraction of compounds within a large spectrum of polarities such as phenolics antioxidants because it provides high efficiency in short time.

The extracted compounds were separated by HPLC-UV-ELSD analysis which revealed the presence of different compounds useful for food, cosmetic of pharmaceutical purposes.

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