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Cloud point method applied for the Apolipoprotein A-I extraction from human plasma and its identification by tandem mass spectrometry

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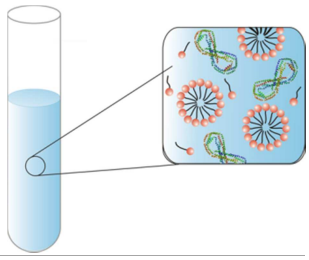
ABSTRACT

This work describes the extraction of the Apolipoprotein A-I (ApoA-I) from human plasma using the cloud point extraction (CPE). The CPE was carried out with a nonionic surfactant (5% w/v Triton® X-114), and the presence of a salting-out effect (10% w/v NaCl) promoted biocompatible separation conditions at room temperature and pH 6.8. The ApoA-I present in the surfactant-rich phase was identified by tandem mass spectrometry after two-dimensional gel electrophoresis.



Keywords: Protein extraction; Apolipoprotein A-I; Cloud point; Gel electrophoresis; Mass spectrometry.

1. Introduction

Besides other applications [1,2], surfactants are also employed for the extraction of different biomolecules presenting hydrophobic characteristics [3,4]. They allow selective and efficient separation of proteins, especially membrane proteins, which cannot be easily extracted from sample matrix using milder treatments, exploiting the cloud point (CP) phenomenon [5].

CP phenomenon occurs due to the reduction of the surfactant monomers solubility, which is caused by the increase of ionic strength and/or temperature of the solution [6,7,8], resulting in the aggregation of micelles and formation of two apparently immiscible phases, one presenting high surfactant concentration, termed surfactant-rich phase and other with low surfactant concentration, called surfactant-poor phase [9,10]. Due to differences in polarity, hydrophobic proteins are expected to be present in surfactant-rich phase and the hydrophilic ones in the surfactant-poor phase, if dimensional aspects and the concentration of the protein are not taken into account [3,11].

The most common adopted strategy to promote phase separation when extraction of proteins is desired consists in the adjustment of the ionic strength of the medium by adding kosmotropic ions to the solution, exploiting the salting-out effect. This procedure minimizes protein denaturation allowing phase separation at low temperature [12,13]. The use of nonionic surfactants, which separate phases at low temperatures [14], such as some surfactants from Triton® series, can be another alternative for this purpose [15-17].

Different strategies are recently described in the literature demonstrating the feasibility of cloud point extraction (CPE) for the separation of different classes of proteins at laboratorial or industrial scale [18-21]. The application of nonionic molecules consists is the most widespread alternative and can include separations based on the polarity [9], excluded vol-

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umes [22] or the charge of the proteins [23]. However, few applications are found using complex matrices, such as milk derivates [9,24] and human blood plasma [25].

Particularizing the discussion about blood plasma, this matrix presents proteins with different concentrations ranges and physical characteristics. In this way, the selective removal of specific proteins or classes of proteins consists in a fundamental step in sample preparation procedures applied for several studies, such as proteomics [26,27].The application of an optimized CPE procedure can be an interesting alternative for amphiphatic blood proteins isolation due to the interaction of these macromolecules with the hydrophobic interior of micelles using a simple analytical procedure previously optimized. As a model of amphiphatic blood protein, one can mention Apolipoprotein A-I (ApoA-I) which is found in blood as the major component of high-density lipoproteins (HDL) of plasma [28] being its concentration around 100-150 mg dL-1 [29]. Besides acting in lipid binding and formation of HDL, ApoA-I contributes to cellular cholesterol efflux and the activation of lecithin: cholesterol acyltransferase (LCAT) [23].

In this way, the main goal of this work was to develop a simple, efficient and inexpensive CPE method for ApoA-I extraction from human plasma including the further protein characterization using mass spectrometry to point out the potentialities of CPE for purification of amphiphatic proteins from a complex matrix.

2. Material and methods

2.1 *Reagents and samples.*

The nonionic surfactant polyethylene glycol tert-octylphenyl ether (Triton® X-114) was obtained from Sigma-Aldrich (Steinheim, Germany). NaCl, KH2PO4/NaOH buffer and acetone were of analytical grade (Merck, Darmstadt, Germany or J.T. Baker, Phillispsburg, USA). The reagents for electrophoresis were from Amersham Biosciences (Uppsala, Sweden), and the mass spectrometry grade trypsin was from Promega (Madison, USA). All solutions were prepared with deionized water (≥ 18.2 MΩ cm) using a Milli-Q water purification system (Millipore, Bedford, USA).

Human plasma was supplied by the Clinical Hospital of Unicamp, which promoted the control of the absence of disease. The bags of plasma were received in our laboratory with a seal of approval. The plasma was sonicated for 15 min and aliquots of 15 mL were frozen at –18 °C in separated flasks. During the experiments, defrosted aliquots were never refrozen to avoid protein precipitation.

2.2 *Phase separation procedure and surfactant removal.*

Sodium chloride (0.83 g) was dissolved using a vortex mixer in glass tubes containing 8 mL of 5 % (w/v) Triton® X-114 and 200 µL of 0.1 mol L^{-1} KH_2PO_4 / NaOH at pH 6.8. A volume of 100 µL of plasma was then added, the mixture was homogenized again, and the glass tubes were centrifuged at 1780 g for 10 min to accelerate phase separation. The upper and the lower phases were called surfactant-rich and poor phases, respectively. The temperature was ca. 25 °C for all experiments.

Surfactant removal for total protein quantification according to the Bradford´s method [30] was carried out as follows: for quantification of total proteins present in the surfactant-rich phase, 1 mL of the phase was added to 8 mL of ice-cold acetone in an ice bath, and incubated on ice for 1 h. The supernatant was removed, the pellet dried at room temperature and dissolved into 1 mL of KH_2PO_4 / NaOH buffer at pH 6.8. Proteins contained in the surfactant-poor phase were directly quantified without removing the surfactant, since that its concentration was considered negligible [5].

After protein quantification, partition coefficients (K_p), defined as the ratio between protein concentration in surfactant-rich and -poor phases, respectively, were determined for each studied system to obtain favorable ApoA-I extraction conditions.

2.3 *Gel electrophoresis separation.*

Sodium dodecyl sulphate gel electrophoresis (SDS-PAGE) was used to evaluate protein fractions present in the surfactant-rich and -poor phases. For protein precipitation, 8 mL of ice-cold acetone was added to 1 mL of the surfactant-rich phase in an ice bath. This mixture was then incubated for 1 h. Then, the supernatant was removed, the precipitate dried at room temperature and dissolved into 1 mL of 0.05 mol L^{-1} Tris-HCl buffer containing 13.6 % (w/v) glycerol, 2.7 % (w/v) SDS, and 5.4 % (v/v) β-mercaptoethanol at pH 6.8. The surfactant-poor phase (500 µL) was directly diluted with 500 µL of the same buffer solution. SDS-PAGE separation employed a lab cast 10 % (w/v) polyacrylamide gel of 1.5 mm (Amersham Biosciences, Uppsala, Sweden). A constant voltage of 90 V and 25 mA were applied overnight for protein separation. Protein masses of 5.2 and 10.5 µg were applied to each lane, considering the surfactant-rich and -poor phases, respectively. The protein marker (#SM0431, MBI Fermentas, Hanover, USA) was used for molar mass estimation.

The presence of ApoA-I in the surfactant-rich phase was also checked by two-dimensional gel electrophoresis (2D-PAGE). For this task, 20 mL of this phase was precipitated with acetone following the precipitation procedure already described. The precipitate was dissolved in a mixture containing 7 mol L^{-1} urea and 2 mol L^{-1} thiourea, and immediately desalted using a PD-10 column (Amersham Biosciences, Uppsala, Sweden) containing SephadexTM G-25 for removing the excess of phosphate, chloride and sodium. Finally, the desalted solution was lyophilized and dissolved using a buffer containing 7 mol L^{-1} urea, 2 mol L^{-1} thiourea, 2 % (w/v) CHAPS, 0.002 % (w/v) bromophenol blue, 0.5 % (v/v) ampholytes (pH 3-10) and 1 % (w/v) DTT. A 250-µL buffered sample was kept overnight in contact with a 13-cm IPG strip (ImmobilineTM DryStrip, Amersham Biosciences, Uppsala, Sweden, pH 3-10). An isoelectric focusing procedure was carried out in a Multiphor II system (GE Healthcare) totalizing 16000 V h. Then, proteins were reduced and alkylated

according to Garcia et al. [31] followed by separation in the second dimension using the same procedure already described for SDS-PAGE.

The gels obtained were stained with colloidal CBB G-250 [32] and scanned using an ImageScanner II (Amersham Biosciences, Uppsala, Sweden) with the densitometer operating at 300 dpi. The software Gel-Pro Analyzer 3.1 (Media Cybernetics, Maryland 20910, USA) was used for analyzing the bands in the SDS-PAGE, and the ImageMaster 2D Platinum 6.0 software (GeneBio, Geneva, Switzerland) was employed for data treatment of the separation by 2D-PAGE.

2.4 *Tandem mass spectrometry analysis.*

Spots manually removed from the gel were digested using a micro SPE plate containing a peptide affinity resin – Montage® In-Gel digestZP kit (Millipore, MA, USA), according to the manufacturer's recommendations.

The digested proteins were analyzed using the dried droplet method [33]. For this task, 1 μL of each sample was mixed with 1 μL of MALDI matrix (1 % w/v α-cyano-4-hydroxycinnamic acid dissolved into 1:1 v/v acetonitrile: water solution, containing 0.1 % v/v TFA). The mixture was spotted to a MALDI plate and dried at room temperature until complete solvent evaporation.

Mass spectra were acquired in a MALDI-Q-TOF Premier mass spectrometer (Waters – Micromass, Manchester, UK), and obtained in the positive mode using a Nd:YAG laser (337 nm) MALDI source. LockMass correction was performed with PEG 800 in the lock mass spot. Argon was used as collision gas and a typical collision energy (34 - 161 eV) was employed. The instrument was controlled by MassLynx 4.1 software.

Peptide mass data were analyzed for corresponding protein matching in the MSDB database with oxidation of methionine as variable modification, carbamidomethylation of cysteine as fixed modification, ± 0.1 Da peptide and fragment mass tolerance, and a maximum of one missed cleavage site setting on the MASCOT Server database search engine (Matrix Science, London, UK) [34]. The significance threshold was set at $P < 0.05$, corresponding, in this case, to a minimum MASCOT score of 47. Additional confirmation of protein molar mass based on gel electrophoresis experiments was performed.

3. Results and Discussion

Effect of Triton® X-114 concentration.

The Figure 1 shows the partition coefficients (K_p) as a function of Triton® X-114 concentration (2-15 % w/v). Variations in the K_p values (0.21 ± 0.02 and 0.57 ± 0.05 for 2 and 15 % w/v Triton® X-114, respectively) were observed when the Triton® X-114 concentration increased by a factor of ca. seven. Considering the difficulty to manipulate a 15 % (w/v) Triton® X-114 solution (the higher K_p value observed) due to its high viscosity, and due to the K_p value remained almost constant between 5 and 10 % (w/v), a 5 % (w/v) Triton® X-114 concentration was then used as the optimal condition.

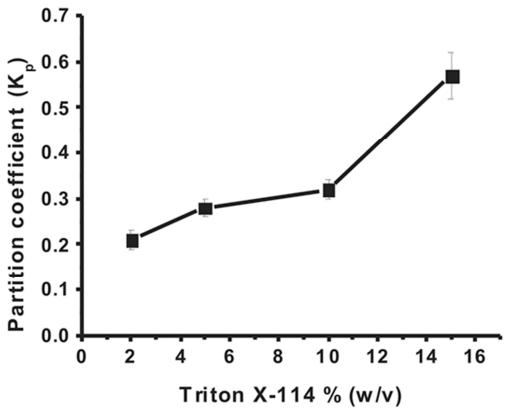

Figure 1. Partition coefficient as a function of Triton® X-114 concentrations using 2 mL of sample volume, 10 % (w/v) NaCl, 20 min of contact time and pH 7.2.

Proportion of volume between sample and surfactant solution.

According to the Figure 1, the partition coefficient increased when surfactant concentrations were raised. However, as commented before, the highly viscous surfactant solution is difficult to handle, while 5 % (w/v) Triton® X-114 was possibly not enough to extract higher quantities of protein contained in a certain volume of the human plasma. In the Figure 1, for example, the results were obtained using 2 mL of sample volume. Thus, the total protein mass was changed, ranging sample volumes from 50 to 2000 μL, keeping the surfactant concentration constant. These experiments allowed determining the proportion between sample volume and solution volume of the surfactant. The proportions 1:160, 1:80, 1:40, 1:16, 1:8 and 1:4 related to sample volume: surfactant solution were evaluated. By decreasing this proportion, a decrease in the partition coefficient was observed from 0.62 ± 0.02 to 0.30 ± 0.01 when 1:80 and 1:4 were respectively used (results not shown). Protein aggregation at higher concentrations can be happen, decreasing the migration of proteins to the surfactant-rich phase. Then, 1:80 was fixed as sample volume:surfactant solution proportion.

Effect of NaCl concentration.

The effect of electrolytes on phase separation of nonionic surfactants is well known. The electrolyte salts out the polyoxyethylated surfactants by dehydration [35,36], decreasing the cloud point temperature. In this way, NaCl concentrations were evaluated from 6 to 12 % (w/v) range and two phases were visually formed at room temperature (25 °C).

The Figure 2 shows the partition coefficient (K_p) as a function of NaCl concentration. It was observed an increase in K_p as NaCl concentration was raised, a constant behavior between 10-12 % (w/v). This behavior can be explained due to the volumes of surfactant-rich phases obtained after the two phase separations. When the NaCl concentration was increased, smaller volumes of surfactant-rich phase (2.5 mL at 6 % and 1.2 mL at 12 % w/v) were observed (results not shown). For small surfactant-rich phase volumes, it was supposed that the proteins are distributed into a small volume,

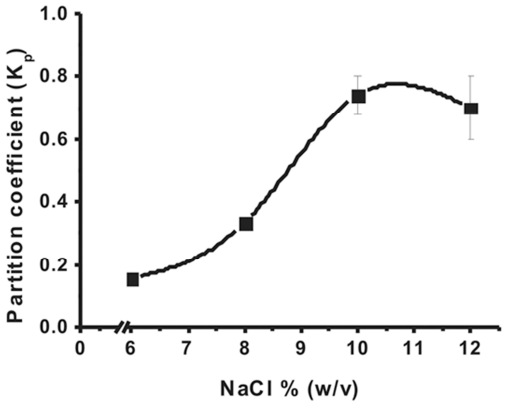

Figure 2. Partition coefficient as a function of NaCl concentration using 5 % (w/v) Triton® X-114, 100 µL of sample volume, 20 min of contact time and pH 7.2.

the preconcentration of the proteins being naturally obtained, and the partition coefficient value increased. In this context, because of a constant behavior of K_p above 10 % (w/v) NaCl, this concentration was used for subsequent separations.

Effect of contact time and pH.

After mixing all reagents, the partition coefficient remained constant from 0 to 30 min evaluated range, demonstrating that the extraction equilibrium was quickly attained. This results is according to the literature, where only slight variations in the alcohol dehydrogenase activity were found when increasing the contact time from 1 to 11 min [37], and from 10 to 50 min for albumin extraction [20]. Thus, a shorter contact time between protein and surfactant aggregates is preferable.

Few reports in the literature using the cloud point method [20,38,39] describe the influence of the pH value on protein extraction. On the other hand, most protein extraction methods are pH dependent. The pH can change the charge of chemical groups present on side chains of the amino acids, modifying the net global charge of the protein. The number of charged groups on the protein molecule surface varies by changing the pH, which implies in the modifications in protein structure [40]. This behavior also changing surfactant micelles and protein interactions and, consequently, the partition coefficient.

Saitoh and Hinze [41] reported hydrophobic membrane protein extraction over pH 5.5 to 6.5 using a zwitterionic surfactant C9-APSO4; however, hydrophilic proteins were not extracted at this pH range. In the present work, the effect of pH was then evaluated over the physiological pH range (6.4 – 7.4) with the goal in maintaining the protein structure. The partition coefficients ranged from 0.49 ± 0.08 for pH 6.4 to 0.33 ± 0.05 for pH 7.4, respectively. Because the small variation of K_p in the pH range studied, the process was carried out at pH 6.8 (K_p = 0.48 ± 0.04).

PAGE profile and ApoA-I identification.

Figure 3 shows the bands of the proteins present in the surfactant-rich and -poor phases, after the optimized extraction by cloud point (5 % w/v Triton® X-114, 100 µL of sample volume, 10 % w/v NaCl, contact time < 1 min and pH 6.8). According to this figure, surfactant-rich phase did not present protein bands at molar masses higher than 100 kDa (see lane 3), indicating a poor interaction between the surfactant micelles and these proteins. Two bands (lane 3) were observed in the surfactant-rich phase. The R2 band may be attributed to albumin (ca. 66 kDa), which presents high concentration in human plasma (51-71 % of total protein) [42], contributing to its extraction to this phase. Even considering that albumin presents remarkable hydrophilic characteristics, its presence in the surfactant-rich phase is inherent to CPE since this phase is somewhat hydrated. The R1 band (ca. 27 kDa) is attributed to the ApoA-I protein, and the densitometric analysis (N=9) reveled that this band presents ca. 174 ± 6 ng of protein. Additionally, this protein can also be observed in the surfactant-poor phase (lane 4).

Mass spectrometry analyses were performed to confirm the identity of ApoA-I present in surfactant-rich phase after separation using two-dimensional gel electrophoresis. In this case, the success on protein characterization depends on the adopted separation strategy. In this way, the use of a multidimensional separation system avoids the overlap of different protein bands in the same region of the gel and allows adequate correlation with proteins contained in protein databanks.

Two-dimensional electrophoretic profile obtained for the proteins contained in surfactant rich-phase is shown in Figure 4. Analysis of spot A allowed identification of three peptides, described in Table 1, which had their primary sequence

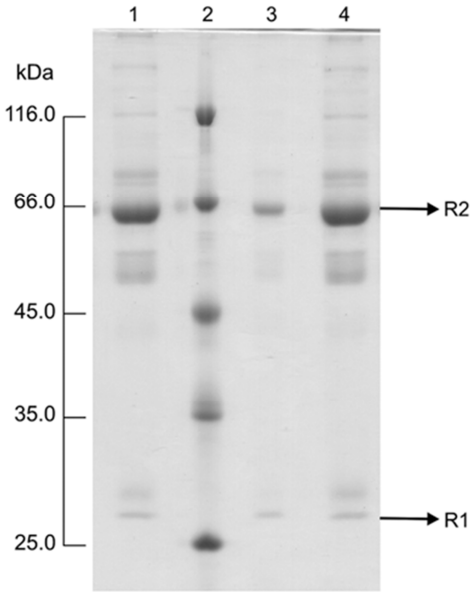

Figure 3. SDS-PAGE of proteins fractions obtained with the optimized conditions using the cloud point extraction. Lane 1 = plasma matrix without phase separation; Lane 2 = standard molar mass markers; Lane 3 = surfactant-rich phase (5.2 µg); Lane 4 = surfactant-poor phase (10.5 µg). Albumin and ApolipoproteinA-I are represented by R1 and R2, respectively.

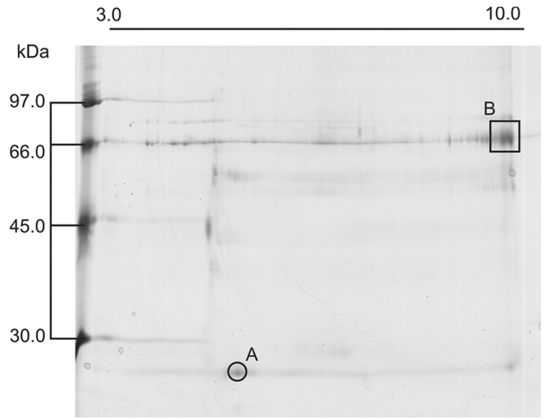

Figure 4. Separation of the proteins present in the surfactant-rich phase by two-dimensional gel electrophoresis. Spot in region A indicates ApoA-I protein, while region B represents albumin.

revealed by tandem mass spectrometry, through spectra shown in Figure 5. In fact, those three identified peptides correlate with ApoA-I protein (access code CAA00975), correspond to a coverage of 12% of the primary structure of the protein (with a MASCOT score of 97). The theoretical mass (28061 Da) obtained from MSDB databank is in accordance to the experimental value obtained after 2D-PAGE separation (ca. 26650 Da), indicating that the variation between theoretical and experimental masses is lower than 5%.

4. Concluding remarks

An alternative method for ApoA-I extraction from human plasma based on a cloud point strategy was developed. Biocompatible conditions were used for ApoA-I separation, such as room temperature and pH 6.8, with 5 % (w/v) Triton® X-114 and 10 % (w/v) NaCl. At optimized conditions, the partition coefficient (ca. 0.5), calculated from the total protein concentration, indicates a relatively low affinity of the human plasma proteins and surfactant aggregates. However, the electrophoretic separation by 2D-PAGE related to those proteins in the surfactant-rich phase presented a clear gel, identifying the presence of the target protein (ApoA-I), which was successfully extracted to this phase. The band of ApoA-I was also observed in the surfactant-poor phase through SDS-PAGE analysis. The small amount of the surfactant present in the poor phase can explain this behavior.

The similarity of this procedure with that one previously published [14], demonstrates its robustness, what should be, in our opinion, the target of any analytical proteomic approach.

Table 1. ApoA-I peptides identified by tandem mass spectrometry.

Experimental m/z value	Experimental molecular mass (Da)	Theoretical molecular mass (Da)	Peptide
1012.5754	1011.5681	1011.5713	K.AKPALEDLR.Q
1226.5383	1225.5310	1225.5364	-.DEPPQSPWDR.V
1301.6343	1300.6270	1300.6411	R.THLAPYSDELR.Q

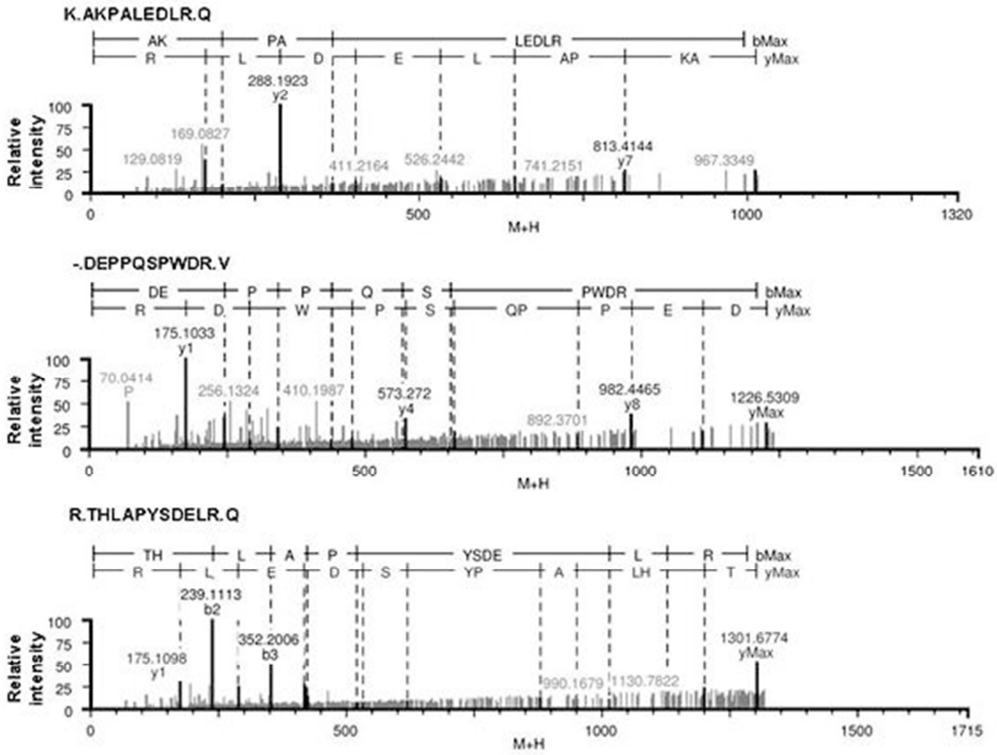

Figure 5. Fragment ion spectra and fragmentation profile obtained for identified peptides shown in Table 1.

Finally, the procedure can easily be scaled-up, employing a simple, fast (ca. 15 min) and low cost method (US$ < 0.50 per sample) for ApoA-I separation. Additionally, it can be considered a less aggressive process compared to traditional methodologies due to the use of nonionic surfactants only and simple electrolytes.

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