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Uncertainty contribution of the chromatographic factorsduring an HPLC-UV elution of four vitamers compounds

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ABSTRACT

The influence of the main chromatographic parameters on the conduct of the HPLC-UV assays was elaborated on the elution of four vitamers (retinol, retinyl acetate, tocopherol and tocopheryl acetate). Considering, both type A and type B methods to estimate their measurement uncertainty magnitudes, taking into account the specifications information and the repeated available data. In this context, the retention time variability was used to cover the effect of the most factors. Likewise, the areas data for all peaks of interest were randomly generated on current chromatograms by the software, to examine the baseline effect on the peaks integration. As a result, the peak area's variability, which reaches up to 5%, indicates that the uncertainty contribution, in area measurement is mainly due to the baseline effect, which make difficult the peaks integration, especially for low amount compounds. Nevertheless, the low variability between runs in the retention time reflects much more the stability of the HPLC-UV system and attests that the influences of the other chromatographic parameters remain insignificant.

Key words: Uncertainty, chromatographic factors, HPLC-UV assays, area measurement, vitamers

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INTRODUCTION

Sources of uncertainty arising from the chromatographic parameters are difficult to prospect and to implement their evaluation according to the bottom up approach, since there is no model that clearly links all these factors (input quantities) with the measurand (instrument response). Indeed, it is strongly incited to use this approach due to its consistency with the GUM principles "guide for the evaluation and expression of uncertainty in measurement" [1], where the identification of the potential sources of uncertainty is a priority. Nevertheless, the main factors influencing the conduct of the HPLC-UV assays have been already enumerated. However, assessing their contributions requires much caution when using the instrument response variability. Other regulated alternative approaches "Top down", based on the ISO 21748 [2], are used also to evaluate as a global way the measurement uncertainty, but without knowing what factors affect more over the conduct of the analytical process. In all cases, it is often suggested to use the change of the instrument response (peak area) to estimate the measurement uncertainty [3], [4], that is comprehensible for empirical approaches, since it satisfies an overall estimate of the uncertainty. However, it remains to be careful to take this variation as well, to characterize only the influence of the chromatographic parameters. Moreover, the most studied proposals suggest of injecting a same solution several times [5], nonetheless, without ensuring that the ambient environment will not affects these repeated experiments. Whereas, the response variability, when used to represent only the chromatographic parameter influences, must be independent of all the previous steps. Otherwise, there is a higher risk to overestimate their associated contributions. Indeed, the main chromatographic parameters affecting the instrument response precision (peak area) during HPLC-UV assays are the detection (Det), the flow-rate (F), mobile phase composition (mbl_ϕ), column temperature (temp), baseline drift (Bl_dft), baseline noise (Bl_ns) and integration [6], the figure 1a illustrates the related sources of uncertainty to these parameters.

In this context, the valuation of the identified factor effects on the conduct of the HPLC-UV assays was elaborated on the elution of four vitamers (retinol, retinyl acetate, tocopherol and tocopheryl acetate). Using both type A and type B estimations, as reliable and equal methods to evaluate the measurement uncertainty [1], taking into account the specifications information and the available repeated data. When using the manufacturer information's, the detection and the flow-rate contributions can be evaluated according a type B method. Furthermore, the retention time (t_R) repeatability was accounted to complete the uncertainty contributions for many factors, assuming thereby a type A estimation. In fact, retention time is a qualitative quantity that is well independent from the previous handling steps, its precision in our point of view is an important tool to estimate the chromatographic parameter influences during instrumental runs. Since, it depends from mobile phase flow-rate, mobile phase composition, system integration and column temperature changes during the chromatographic trials [6].

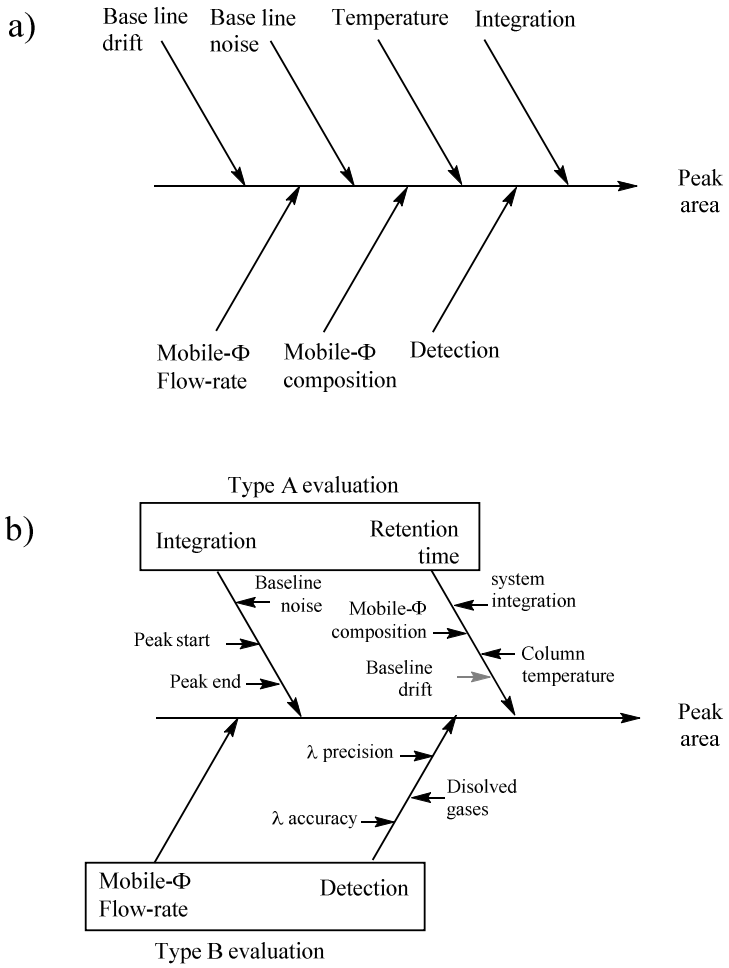

Fig. 1. Identified sources of uncertainty during the chromatographic assays: a) the main influent factors, b) The same identified effects rearranged according the uncertainty estimation type

In addition, post-run analysis mode of the software was performed to generate independent data from existing chromatograms, to use as a correct way the variability of the peak area, for the baseline effect check on the peak integration and to evaluate the uncertainty contribution of this factor. The baseline noise often makes difficult the identification of the beginning and end of the peaks, despite being in its proper form, as it is normally encountered for peaks of low content analytes. However, the baseline drift will have negligible effect, if the experiments will carried out during a short time interval. [4]. All of these influences were rearranged according the estimation type as illustrated by the Ishikawa diagram of the figure 1b.

MATERIALS AND METHODS

Reagent

Retinol, retinyl acetate, tocopherol, tocopherol acetate and BHT (2,4-di-tert.-butyl hydroxyl toluene) were purchased from Sigma-Aldrich (St Louis, USA). Absolute ethanol, Methanol, Acitonitrile and ethyl acetate were CHROMASOLV, HPLC grade solvents from Sigma-Aldrich (St Louis, USA).

HPLC system

A Shimadzu HPLC LC-10Avp series system (Shimadzu japan) constituted of a SCL-10AVP controller, a LC-10ADvp micro-volume double plunger pump, aDGU-14A on-line degasser, a SPD-10AvpUV variable wavelength detector and a FCV-10ALvp low pressure gradient unit, to homogenise the mobile phase a SUS mixer was used. The chromatographic system was controlled by a personal computer using the LCsolution software (Shimadzu) to acquire all kinds of data. The injection device consisted of a Rheodygne 7725i manual injector, equipped with a 20µL loop.

Chromatographic conditions

The mobile phase was a constant composition (70:30, v/v) of Methanol/Acitonitrile, the elution was established at 2 ml/min flow-rate. The separation was achieved at room temperature using an analytical column Zorbax SB-C18 (4.6 x 250mm, 5-µm) from (Agilent) that is protected by a guard column Zorbax SB-C18 (4.6 x 12.5 mm, 5-µm) from (Agilent). Detection was monitored in dual mode at the maximum wavelength 325nm and 292nm respectively, for vitamin A and vitamin E compounds.

Standards and sample preparation

The Standards weresynthetic pure substances (over 98 % purity) of vitamin A and vitamin E, they were mixed to prepare three equidistant levels of working solutions, at the concentration range (0.1 −0.5 µmol L^{-1}) for retinol and (4.5−22.5 µmol L^{-1}) for tocopherol, by diluting an adequate take with a solvents mixture of ethanol/ethyl acetate (1:1, v/v). [7]. containing BHT as an antioxidant and a constant amount of retinyl and tocopheryl esters as internal standards for retinol and tocopherol, respectively.However, the sample was a human transfusion plasma obtained from the paediatrics service of the CHUTlemcen, Algeria. Plasma (50µL) was treated by the same solvent mixture to precipitate the proteins and was spiked with the same amount of the internal standards. The native vitamers were separated mechanically by vigorous shaking and a fasted centrifugation; to obtain a clear supernatant liquid for direct injection in the HPLC system.

Experimental

The experiments were performed as is done for system suitability test [8], using theplasma aqueous dilutionsand the organic standards solution, as typical mixtures for this bioanalytical assays. Duplicate standard solutions at the three concentration levels and six plasma replications were injected for one series HPLC-UV analysis, as it is required for repeatability assessmentsby the referential pharmacopoeias [9−11]. The collected data were then handledto examine the chromatographic parameters effects and to evaluate their related uncertainty contributions.

RESULTS AND DISCUSSION

Figure 2 shows typical chromatograms obtained from HPLC-UV assays of the standards and the samples.As we can see, all the analytes of interest (retinol, retinyl acetate, tocopherol and tocopheryl acetate) were well identified and no interference was observed from the BHT. However, the resolution of the peaks leads easily to implement a suitable evaluation.

Uncertainty evaluation

The identified uncertainty components of the chromatographic step were estimated individually, by means oftype A and type B methods.

Flow-rate

Specifications on the instrument indicate a flow rate precision and accuracy within ±0.3 % and ± 2 %, respectively, therefore type B evaluation was used for this parameter, assuming a triangular distribution, there:

$$\sigma(\mathcal{F})_{\text{precision}} = \frac{0.003}{\sqrt{6}} = 1.225 \times 10^{-3}\ \text{mL} \cdot \text{min}^{-1}$$

$$\sigma(\mathcal{F})_{\text{accuracy}} = \frac{0.02}{\sqrt{6}} = 8.165 \times 10^{-3}\ \text{mL} \cdot \text{min}^{-1}$$

The standard uncertainty relating the mobile phase flow-rate was then:

$$u(\mathcal{F}) = \sqrt{(1.225 \times 10^{-3})^2 + (8.165 \times 10^{-3})^2} = 8.2564 \times 10^{-3}\text{ml} \cdot \text{min}^{-1}$$

The relative standard uncertainty for all compounds becomes then:

$$\frac{u(\mathcal{F})}{\mathcal{F}} = \frac{8.2564 \times 10^{-3}}{2} = 4.1282 \times 10^{-3}$$

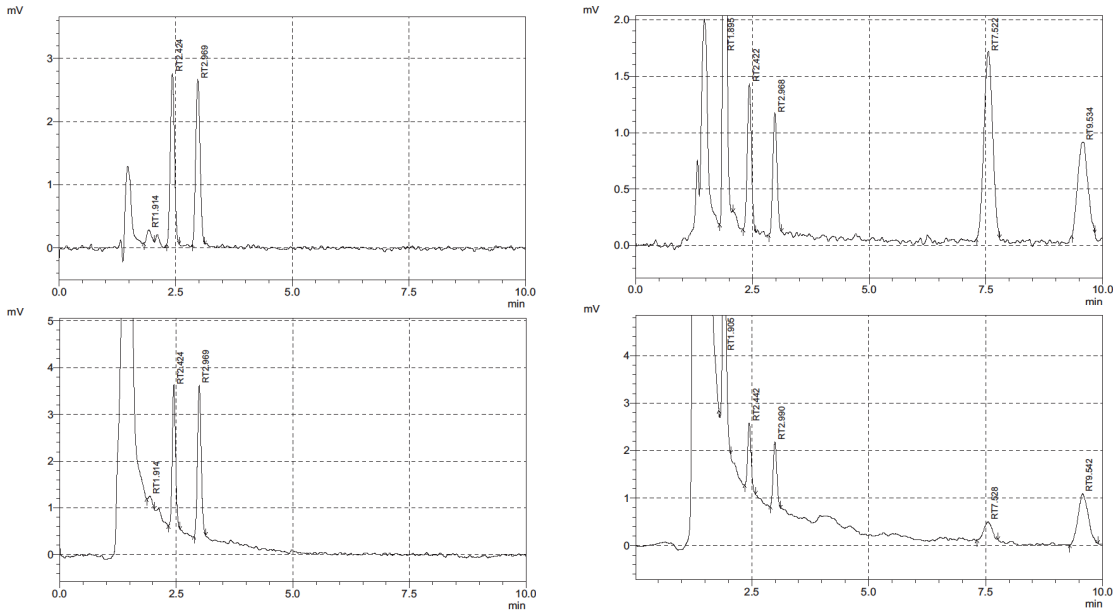

Fig. 1. HPLC-UV response obtained in dual mode; 325 nm (left) and 292 nm (right) after injecting: a standard solution (top chromatograms) and a treated plasma sample (Bottom chromatograms).Identified peaks: BHT (t_R = 1.9 min), Retinol (t_R = 2.4 min), Retinyl acetate (t_R = 3.0 min), tocopherol (t_R = 7.5 min), tocopheryl acetate (t_R = 9.5 min).

Detection

The detection errors in the HPLC techniques coupled with UV–visible detectors can arise from wavelength accuracy [12] and gases dissolved in the mobile phase [13]. There are no information about the dissolved gases, however, their presence in the mobile phase increase the influence of the random noise and the drift, accordingly, this effect was accounted in baseline contribution. However, the manufacturer specifications guarantee a wavelength functioning with ± 1 and ± 0.1 nm,respectively for accuracy and precision. We consider a type B evaluation and assuming for this information a triangular distribution, then:

$$\sigma(\lambda)_{\text{precision}} = \frac{0.1}{\sqrt{6}} = 0{,}0408 \text{ nm}$$

$$\sigma(\lambda)_{\text{accuracy}} = \frac{1}{\sqrt{6}} 0{,}4082 \text{ nm}$$

The standard uncertainty for the detection was thereby:

$$u(\text{Det}) = u(\lambda) = \sqrt{(0{,}0408)^2 + (0{,}4082)^2} = 0.41024 \text{ nm}$$

The relative standard uncertainty of this parameter becomes for each compound as follows:

$$\left(\frac{u(\lambda)}{\lambda}\right)_{Retinol} = \frac{0.41024}{325} = 1.2623 \times 10^{-3}$$

$$\left(\frac{u(\lambda)}{\lambda}\right)_{Retinyl} = \frac{0.41024}{325} = 1.2623 \times 10^{-3}$$

$$\left(\frac{u(\lambda)}{\lambda}\right)_{Tocopherol} = \frac{0.41024}{292} = 1.4049 \times 10^{-3}$$

$$\left(\frac{u(\lambda)}{\lambda}\right)_{Tocopheryl} = \frac{0.41029}{292} = 1.4049 \times 10^{-3}$$

Retention time variability

The table 1 express the statistical evaluation of the retention time contribution for each compound, using the data obtained from chromatographic assays. Hence, a type A method was applied and the standard uncertainty was accounted by means of a standard deviation.

Table 1: Evaluation of the retention time contribution from run to run variability for the standards and the samples during HPLC-UV assays

	A Vitamers		E Vitamers	
	Retinol	Retinyl	Tocopherol	Tocopheryl
Standard	2.4137	2.9613	7.5235	9.5503
	2.4123	2.9568	7.4836	9.497
	2.4123	2.9567	7.4534	9.4637
	2.4063	2.9484	7.4157	9.3689
	2.4143	2.9589	7.4522	9.4652
	2.4136	2.9569	7.4481	9.4515
Mean t_R(min)	2.4121	2.9565	7.4628	9.4661
SD x10^{-3}(min)	2.9	4.3	36.7	59.5
RSD (%)	0.12209	0.14686	0.49236	0.62863
Sample	2.4305	2.9757	7.4929	9.4811
	2.4282	2.9737	7.4649	9.4806
	2.4295	2.9743	7.4841	9.4869
	2.4312	2.9754	7.4795	9.4777
	2.4294	2.9728	7.4751	9.4628
	2.4323	2.9760	7.4543	9.4676
Mean t_R(min)	2.4302	2.9746	7.4751	9.4761
SD x10^{-3}(min)	1.5	1.3	13.8	9.1
RSD (%)	0.0603	0.0424	0.1847	0.0960
S_c	0.0023	0.0032	0.0278	0.0426
δ_{tR}%	1.811	1.813	1.240	1.002
$u(\delta_{tR})$%	0.301	0.437	3.717	5.962
$\left(\frac{u(Bl\_dft)}{Bl\_dft}\right)$%	0.754	0.622	0.460	0.529

Baseline drift effect

Baseline drift causes precision errors when it is not constant over a series of analyses, and inaccuracies in peak measurement even when it is repeatable. [14].Figure 2 shows a negative drift in sample chromatograms that is dueto the rapid elution of the polar compounds of the plasma aqueous medium. However, the lack of this drift in the standards chromatograms proves that it is not from an electronic origin,but rather explains a different behaviour of the analytical column, towards the two injected fluids. This is a persistent systematic error, when injecting directly a treated biological fluid, its significance was counted by comparing the absolute bias on the retention time (δ_{tR}), with the standard uncertainty of this bias $u(\delta_{tR})$ [15], if the deviation is significant then:

$$\delta_{tR} = |\bar{t_R}\_spl - \bar{t_R}\_std| > 2u(\delta_{tR}) \quad (1)$$

With:

$$u(\delta_{tR}) = \sqrt{\frac{S_{spl}^2}{n_{spl}} + u^2(\bar{t_R}_{std})} \quad (2)$$

Where:

$\bar{t_R}$_spl ,$\bar{t_R}$_std : The means retention time, respectively for the samples and the standards,

S_{spl}: The standard deviation of the retention time results for sample injections,

n_{spl}: Number of the sample replications,

$u_{(t_R\_std)} = \frac{S_{std}}{\sqrt{n_{std}}}$: is the uncertainty on the mean of the retention time for standards, assumed as the reference value,

The number 2 is the coverage factor at 95% confidence level.

The estimation of the uncertainty contribution due to this effect was carried out, by calculating the pooled standard deviation (Sc) of the equation 3 [16], from the combination of the standard deviations of the retention time results and accounting the bias value in the uncertainty budget [15], using the equation 2:

$$S_c = \sqrt{\frac{(n_{std}-1)S_{std}^2 + (n_{spl}-1)S_{spl}^2}{n_{std}+n_{spl}-2}} \quad (3)$$

$$\frac{u(Bl\_dft)}{Bl\_dft} = \sqrt{\left(\frac{1}{\bar{t_R}\_spl}\right)^2 \left({S_c}^2 + \frac{S_{spl}^2}{n_{spl}} + (\bar{t_R}\_spl - \bar{t_R}\_std)^2 + u^2(t_R\_std)\right)} \quad (4)$$

Where:

n_{std} : is the number of measured values for the standards retention time.

As we can see, the statistical results of the table 1 shows that this deviation is negligible for the vitamin E compounds, since their peak emerge on a horizontal line at 292 nm, against it is significant for vitamin A compounds, where their peaks are the most exposed to this inclination.

Baseline noise effect

The baseline noise effect, which can reflect the imprecision of the peaks integration [14] was evaluated using type A method, by leading the software to detect randomly the start and the end of the peaks in the standard and in the sample chromatograms. This was done through post-run analysis mode, by making simply for the same setting parameters, an automatic execution of the data processing integration of the peaks, while the automation was initialized different times according each standard injection. The table 2 reports the statistical valuation of the data deduced from twelve executions. However, it will be noticed that in all cases the peaks should be visualized for an eventual manual intervention, to eliminate inexact integration due to this automation.

Table 2:Area variability from data generated randomly by the software

	A Vitamers		E Vitamers	
	Retinol	Retinyl	Tocopherol	Tocopheryl
Standard	6530	18152	15315	15771
	6357	17751	14708	15771
	6610	17989	15607	14463
	6357	17751	14708	15771
	6348	17626	15666	15429
	6353	17795	15358	15339
Mean Area	6426	17844	15227	15424
S_{std}	115	191	424	508
RSD%	1.783	1.072	2.787	3.296
Sample	12079	18508	3406	16315
	11553	17444	3564	17474
	12505	18396	3304	16551
	11553	17444	3564	17163
	12401	17444	3176	17174
	12413	17989	3528	16361
Mean Area	12084	17871	3424	16840
S_{spl}	436	498	159	491
RSD%	3.607	2.789	4.641	2.917

Effectively, the software in many cases can lead to improper integration, especially for peaks of low content analytes. The figure 3 shows the uncertainty contribution during the HPLC-UV runs and compares the magnitudes of its components in terms of relative standard uncertainty (RSU). As we can see the area variability, which reaches up to 5%, indicates that the contribution to uncertainty in area measurement is mainly due to the baseline effect. This influence increases in the case of the samples, that shows the drawback of their complex medium when injected directly and where the polar components involve a visible slope in the sample chromatogram of the figure 1, compared to the standards medium, which consists of pure organic solvents. Indeed, changes in the baseline make difficult the identification of the beginning and the end of the peaks, which affect the area measurement, especially for analytes of low contents.

While an excessive fluctuations of the baseline, will certainly lead to the loss of the peak area at its widest part [14].

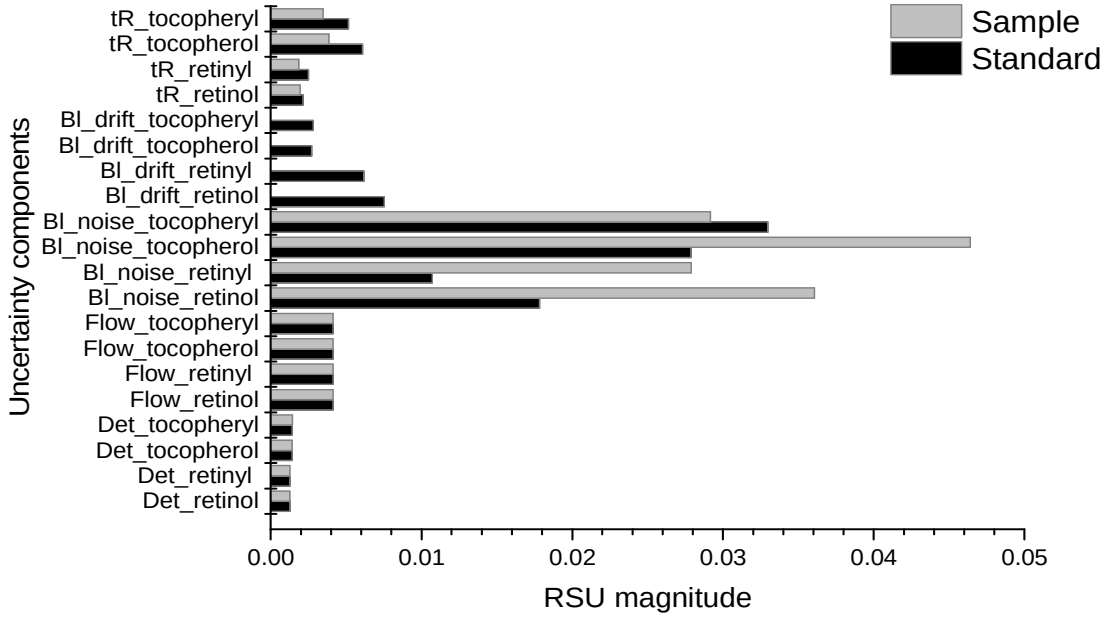

Fig.3. comparison of contributions for the identified uncertainty components

This will be more important for the closing eluted compounds of vitamin E, which peaks are broader at their bases, due to the isocratic elution mode. However, the uncertainty contribution of the drift remains rather small compared to the magnitude of the other chromatographic factors. Yet, the low variability of the retention time reflects much more the stability of the system during the HPLC-UV runs and attests that the influence of the main chromatographic parameters remains insignificant.

CONCLUSION

The influence of the chromatographic parameters such as, mobile phase-flow rate, detection as well as those covered by the variability of retention time, remains insignificant on the conduct of the HPLC-UV assays, since their variability does not exceed 0.8 %. While the baseline effect on the peak area measurement was well revealed, without the need for extra onerous experiments, by the implementation of the post-run integration using the software, which follows only, the random pace of the baseline fluctuations, to identify the beginning and the end of the peak.

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