ing accurate quantification and complete automation. In particular, the profiles were obtained in 7 min, including rinses, which is competitive to HPEC systems. Moreover, capillary electrophoresis systems are robust and could replace HPLC in clinical chemistry analyzers. Moreover, capillary electrophoresis systems, in particular, the profiles were obtained in 7 min, including accurate quantification and complete automation. In

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The monitoring of glycemic control in diabetic subjects has been greatly improved by the development of analytical methods for the measurement of glycohemoglobin concentrations, which reflect average serum glucose concentrations 4–8 weeks preceding the analysis. In particular, because HbA1c is considered one of the most powerful markers in such evaluations (1), optimized conditions for its determination still remain one of the major goals of clinical chemistry, as recently emphasized by Miedema (2).

The development of methods without the limitations inherent in chromatographic, electrophoretic, and immunological procedures (3) is certainly of great interest. These methods may be used to measure HbA1c directly and/or to determine the extent of glycation of the “universal calibrator”. Choosing between these two different approaches is necessarily related to the feasibility (and to the price/performance ratio) of each method. In this context, Roberts et al. (4) recently showed the potentialities of electrospray ionization mass spectrometry (ESI/MS) for quantifying glycohemoglobin. ESI mass spectra from the analysis of whole blood samples were used to evaluate the percentage of glycohemoglobin directly, and comparison of the ESI data with those from established affinity chromatographic procedures showed a particularly good linear relationship. These evaluations were based on the four ionic species detected in ESI conditions after baseline subtraction, i.e., glycated and nonglycated \( \alpha \) - and \( \beta \)-globins.

A different, highly specific approach for unequivocal HbA1c evaluation, which was proposed recently by Kobold et al. (5), is based on enzymatic cleavage of hemoglobin by an endopeptidase. This treatment yielded the \( \beta \)-N-terminal peptides of HbA1c and HbA0, and their separation and quantification was performed by ESI/MS and capillary electrophoresis. This procedure may be used as a reference method for HbA1c measurement. In previous investigations (6–10), we demonstrated that matrix-assisted laser desorption/ionization (MALDI) (11) MS is a valid analytical tool for determining the extent of glycation of in vivo and in vitro glycated proteins.

The MALDI technique is based on the interaction of a laser beam with a solid state sample composed of a
suitable matrix (99%), in which the sample of interest is dissolved (1%). The interaction leads to desorption and ionization of the molecule under study, through the action of reactive species originating from the matrix. MALDI/MS is used mainly for qualitative analyses because ionic species that are very different in mass usually show different yields in ionization as well as in detection. However, for species with mass values of the same order of magnitude, the method may also be applied successfully to quantitative analyses. As emphasized by Jespersen et al. (12), a linear response is found between the measured MALDI peak height ratio and the applied amount of the analyte when an appropriate internal calibrator is used. In the case of glycated globin analysis, no internal calibrator is needed. In fact, the abundant peaks produced by nonglycated species may be used as valid internal calibrants. The good results already obtained in the case of glycated globins (10) are a good example of this aspect.

Twenty healthy subjects (mean age $\pm$ SD, 58 $\pm$ 5 years) and 30 non-insulin-dependent diabetic patients (mean age $\pm$ SD, 63 $\pm$ 6 years; mean disease duration $\pm$ SD, 12 $\pm$ 5 years) were studied. Diabetes was diagnosed according to National Diabetes Data Group recommendations. Metabolic control was assessed by evaluating fasting plasma glucose [mean, 900 $\pm$ 40 mg/L (90 $\pm$ 4 mg/dL) for healthy subjects and 1960 $\pm$ 670 mg/L (196 $\pm$ 67 mg/dL) for diabetic patients] and HbA$_1c$ (13) (mean, 5.5% $\pm$ 0.5% for healthy subjects and 8.8% $\pm$ 1.7% for diabetic patients). All subjects gave their informed consent to the study, which was performed in accordance with the Helsinki declaration of 1975.

Globin was prepared according to Anson and Mirsky (14).

HbA$_1c$ values for healthy and diabetic subjects were measured by an HPLC method (13), performed by the DIAMAT automatic analyzer according to the manufacturer’s recommendations (Bio-Rad Laboratories).

MALDI (11) measurements were performed on a REFLEX$^{\text{TM}}$ (Bruker-Franzen Analytik) time-of-flight instrument equipped with a SCOUT ion source, in the operative conditions described previously (10). Mass spectra were obtained by averaging 20 laser shots; three separate and independent MALDI measurements were made for each sample to evaluate the reproducibility ($\pm$ 5 Da). External calibration, controlled daily, was provided by the [M + H]$^+$ and [M + 2H]$^{2+}$ ions of myoglobin at m/z 16952 and 8476, respectively. Mass accuracy in the range 0.5–0.1% was usually achieved.

The percentages of glycated and nonglycated $\alpha$- and $\beta$-globins were calculated by an automated integration procedure on the corresponding peaks. The imprecision (CV) of these quantitative data was determined by 10 analyses of 10 different samples for which the HbA$_1c$ values ranged from 4.4% to 8.7%. The single day CV was 5%, whereas it increased to 7% for measurements performed over a period of 15 days.

Gridless delayed extraction (15) MALDI-MS analyses were performed with a REFLEX II (Bruker-Franzen Analytik) instrument operating in positive linear mode, using dihydroxyacetophenone as matrix and following the procedure of Pitt and Gormann (16).

The typical MALDI spectrum of the globin fraction of a

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**Fig. 1.** MALDI spectra of globin samples from a healthy subject, obtained without (A) and with (B) gridless delayed extraction, and (C) percentages of the whole pool of glycated products of $\alpha$- and $\beta$-globins obtained by MALDI vs related HbA$_1c$ values. ■, diabetic subjects; △, healthy subjects.
healthy subject is shown in Fig. 1A. The spectrum is very simple because the MALDI technique produces preferentially protonated molecules through ion-molecule reactions occurring in the selvage region close to the solid-state sample. The four peaks composing the spectrum are easily assigned on the basis of their m/z values alone. Thus, peaks at m/z 15127 and 15868 correspond to protonated α- and β-globin molecules respectively; the less abundant peaks at m/z 15289 and 16030 correspond to protonated molecules produced by the condensation of one glucose molecule (180 Da) on the α- and β-globins, respectively (α-globin + glucose - H₂O → 15127 + 180 = 15289 Da; β-globin + glucose - H₂O → 15868 + 180 = 16030 Da). These results are in agreement with the ESI results: both techniques demonstrate that both globins are glycated to a similar extent.

Under ESI conditions, the higher resolution also allows the identification of [M + Na]⁺ and [M + K]⁺ ions (4). Under MALDI conditions, these species are unresolved from the [M + H]⁺ ions. However, when the delayed extraction method (15), which is not available in our laboratory, is used, the same sample from a healthy subject leads to the spectrum shown in Fig. 1B, composed of the same ions and new ones produced by [M + Na]⁺ and [M + K]⁺ species (m/z 15149 and 15165 for α-globin, and m/z 15890 and 15906 for β-globin).

Quantitative data can be obtained easily by a simple automatic integration procedure of the various peaks in the spectra. Data from the 20 healthy subjects and the 30 diabetic patients were obtained by integrating the peak area; it must be emphasized that, in the case of MALDI measurements, the abundance of a ionic species is related to peak area and not to peak height, as is usual in other mass spectrometric measurements (e.g., ESI). The measured area is proportional to the ion current produced by the different species: the sum of the areas of the various detected species represents the total amounts of nonglycated and glycated α- and β-globins ionized by MALDI. This total area is established as 100, and the amounts of the various species are expressed as percentages of it. The low resolution of MALDI measurements carried out without delayed extraction would not affect, in principle, quantitative measurements. In fact, integration of the area related to the nonglycated α- and β-globin chains includes the [M + Na]⁺ and [M + K]⁺ ions revealed in high-resolution conditions, which represent nonglycated species.

In diabetic patients, as expected, a clear increase in the abundance of both glycated components is generally observed.

Linear regression analysis of the percentages of the whole pool of glycated proteins vs HbA1c leads to the straight line shown in Fig. 1C, which crosses the origin of the axis, although its slope is 0.66 and not 1, as is expected. This indicates the different response factor of MALDI and HbA1c in experimental measurements.

In conclusion, the above data show that MALDI/MS may be applied validly to the identification of glycated α- and β-globins. The technique produces clearly reproducible “fingerprints” of globin species, in particular when gridless delayed extraction is available. In our opinion, at this stage MALDI/MS cannot be proposed as a routine tool for HbA1c measurements, mainly because the sample preparation phase is not automated, whereas it is in the proposed ESI-based method. However, it is a valuable tool for the quality control of HbA1c measurements carried out following other principles and may be applied to their standardization.

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