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Exploring Metallodrug–Protein Interactions by ESI Mass Spectrometry: The Reaction of Anticancer Platinum Drugs with Horse Heart Cytochrome c

Angela Casini,[a] Chiara Gabbiani,[a] Guido Mastrobuoni,[b] Luigi Messori,*[a] Gloriano Moneti,[b] and Giuseppe Pieraccini[b]

Since DNA is commonly believed to be the primary target for platinum metallodrugs,[1–4] researchers' interest has mainly focused on the characterisation of platinum–nucleic acid adducts while devoting much less attention to platinum–protein adducts. However, protein-bound platinum fragments probably represent truly active anticancer species—rather than mere drug-inactivation products—provided that metal transfer among distinct binding sites is kinetically allowed.[4] Moreover, platination of specific side chains, which can affect the function of biologically crucial proteins or enzymes through the formation of tight coordinative bonds, might play a relevant role in the overall mechanism and toxicity of platinum drugs.[5] The state of the art of platinum–protein interactions is described in a few articles and reviews;[6–8] in any case, this issue warrants further experimental work.

Thanks to the latest improvements, electrospray ionisation mass spectrometry (ESI-MS) today represents a very powerful method for exploring metallodrug–protein interactions.[6] Owing to the introduction of "soft" ionisation methods, it is possible to transfer the intact metal–protein adduct—whole, in the gas phase—to determine its molecular mass with high accuracy and, thus, obtain its full molecular characterisation. However, much work is still required for the optimisation and the standardisation of experimental ESI-MS procedures directed at these systems. A great variability in ESI-MS responses is generally found in the current literature that depends on many factors, such as the nature of the protein, the nature of the metal, the specific solution conditions, the nature of the metal-bound ligands, pH and the kind of buffer.[9] Apparently, the intrinsic "fragility" of the metal–protein coordination bonds represents a major obstacle, often leading to extensive bond cleavage during ionisation and to loss of chemical information.

[a] Dr. A. Casini, Dr. C. Gabbiani, Prof. L. Messori
Department of Chemistry, University of Florence
Via della Lastruccia 3, 50019 Sesto, Fiorentino (Italy)
Fax: (+39) 055-457-3385
E-mail: luigi.messori@unifi.it

[b] Dr. G. Mastrobuoni, Prof. G. Moneti, Dr. G. Pieraccini
Mass Spectrometry Centre, University of Florence
Via U. Schiff 6, 50019 Sesto Fiorentino (Italy)

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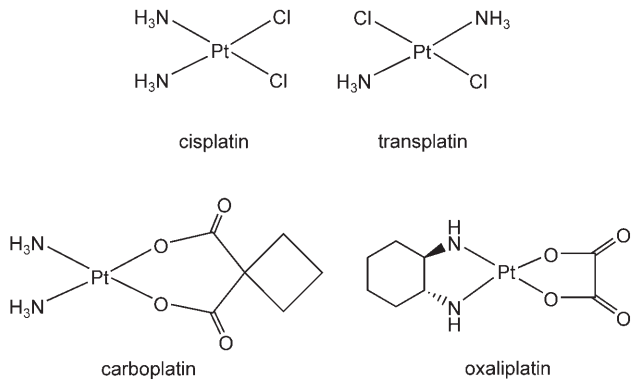
Some pioneering ESI-MS studies of platinum–protein interactions were reported a few years ago by Gibson and co-workers, who used either ubiquitin or myoglobin as model proteins.[10] A number of platinum–protein adducts were identified and characterised in detail. Afterwards, a few additional ESI-MS studies of various metallodrug–protein adducts were reported by other research groups.[11]

Cytochrome c is a small electron-carrier heme protein, localised in the mitochondria, that plays a crucial role in apoptotic pathways.[12] Cytochrome c is also known to be an excellent ESI-MS probe and has been the subject of a number of investigations.[13] This led us to choose cytochrome c as the model protein for our study. The following classical platinum drugs were selected: cisplatin, transplatin, carboplatin and oxaliplatin (Scheme 1).



Scheme 1. Schematic drawing of selected anticancer platinum complexes.

Cytochrome c presents, indeed, a number of favourable features: it is a small protein suitable for ESI-MS studies (M_W = 12362), it shows spectroscopically useful and intense absorption bands in the visible region, it possesses a covalently linked heme moiety, it is known to produce well-resolved ESI-MS spectra in the m/z 1000–2000 region,[13] a few "free" sites are available on its surface that may specifically react with transition metal ions, that is, His26, His33 and Met65.

Platinum–protein adducts were prepared as follows. Horse heart cytochrome c (cyt c) was treated at physiological pH with each platinum compound at various metal/protein ratios, as described in the Experimental Section,[14] for different incubation times (24–168 h). After extensive dialysis, the resulting platinum–protein adducts were analysed by visible absorption spectroscopy, inductively coupled plasma optical emission spectroscopy (ICP-OES) and ESI-MS.

UV-visible spectra show that, under aerobic conditions, cyt c is stable in its oxidised state. Addition of the various platinum drugs, even at relatively high molar ratios, did not appreciably modify the main visible bands at 400 and 550 nm (Figure S1 in the Supporting Information); this indicates that the protein chromophore is not affected. Conversely, ICP-OES determinations of platinated cyt c samples suggest substantial association of platinum to the protein, even after extensive dialysis against the buffer (vide infra).

The ESI-MS spectral profiles for cyt c and its platinum adducts are shown in Figure 1, with the respective deconvoluted spectra in Figure 2. Very satisfactory S/N ratios were obtained for all ESI-MS profiles and for the deconvoluted spectra. Under the nondenaturing experimental conditions used here, cyt c shows a spectral pattern that is dominated by the +8 multicharged species;[13] the relative intensities of the adjacent +9 and +7 peaks are in line with previous observations.[13] The obtained molecular mass of 12362 Da closely matches the theoretical mass of cyt c (i.e. apocytochrome c plus the heme group). Treatment with platinum drugs produced, in all cases, the appearance of new ESI-MS features that are diagnostic of the formation of stable platinum–protein adducts. All shown spectral profiles refer to samples treated for 72 h with a threefold excess of Pt drug and then extensively dialysed. Indeed, these conditions afforded a significant degree of cyt c platination (platination levels ranging from 1.2 to 1.5 Pt moles per cyt c were determined by ICP-OES for all platinum-treated samples).

No substantial modifications of the distribution pattern of the multicharged ions were observed in the platinum–cyt c adducts; this implies that the overall protein conformation is not significantly affected. Remarkably, the peak corresponding to non-platinated cyt c is still observed in all cases; this indicates that platination of cyt c, even after 72 h incubation, is not complete. However, the intensity of this peak is found to greatly decrease (until disappearance) as the incubation times or the applied Pt/cyt c ratios are further increased (see, for instance, Figure 3, below).

The deconvoluted ESI-MS spectrum of the cisplatin derivative (Figure 2B) shows an intense multiplet centred at 12589 Da that corresponds to an adduct in which a single $[Pt(NH_3)_2]^{2+}$ fragment is coordinated to the native holoprotein. An additional multiplet is observed around 12816 Da that corresponds to a 2:1 platinum–protein stoichiometry (the latter mass is just the sum of cyt c plus two $[Pt(NH_3)_2]^{2+}$ fragments). Some additional weak signals, observed around 13062 Da, are assigned to an adduct with a 3:1 Pt/protein ratio. Overall, these results point out that cisplatin forms various kinds of cyt c derivatives with platinum–protein stoichiometries ranging from 1:1 to 3:1.

The pattern of the multiplet centred at 12589 Da deserves some further comments. Remarkably, the relative positions and intensities of the four main peaks within this multiplet are nearly identical to those found by Gibson et al. in the case of the cisplatin adduct of ubiquitin.[10] Thus, it is straightforward to assign the peaks at 12626, 12607, 12589 and 12571 Da to protein binding of the molecular fragments $[Pt(NH_3)_2Cl]^+$, $[Pt(NH_3)_2H_2O]^{2+}$, $[Pt(NH_3)_2]^{2+}$ and $[Pt(NH_3)]^{2+}$, respectively. A similar reasoning may be applied to the description of the ESI-MS multiplets observed in the spectra of the other platinum–protein adducts, reported later.

The ESI-MS spectrum of the transplatin derivative, shown in Figure 2C, again evidences a multiplet centred around 12589 Da, similar in shape to that of cisplatin and consistent with the anchoring of a $[Pt(NH_3)_2]^{2+}$ fragment to cyt c. However, at variance with the case of cisplatin, no signs of the 2:1

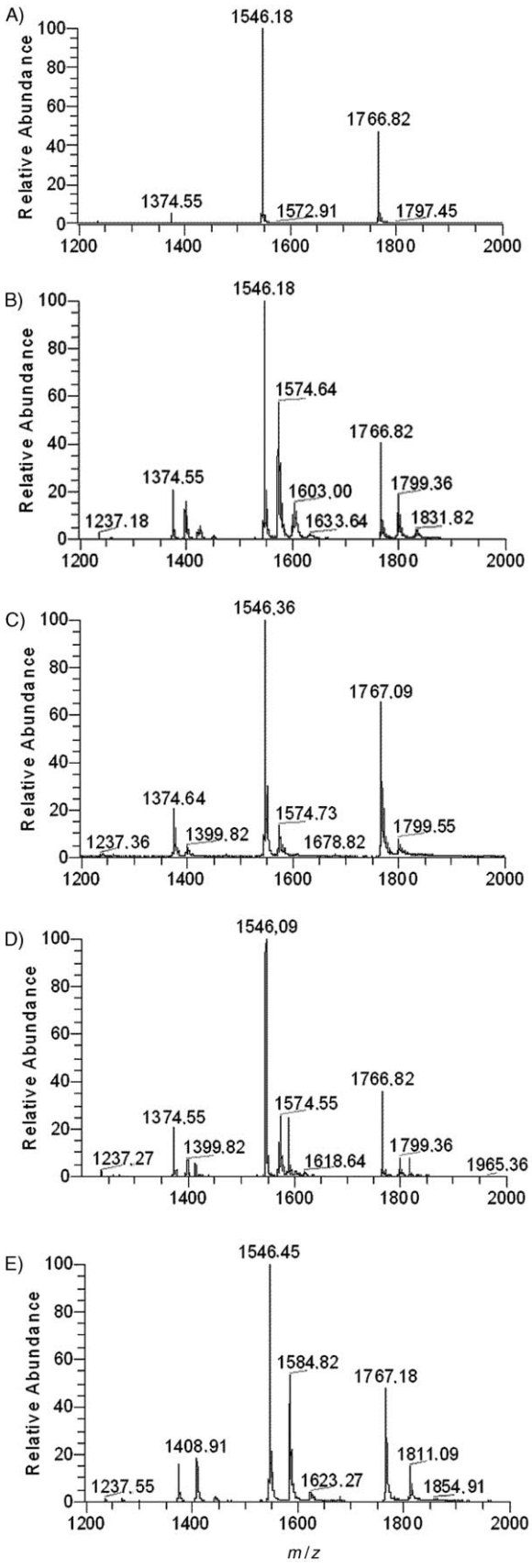


Figure 1. ESI-MS spectral profiles for A) cytochrome c and its platinum adducts: B) cisplatin, C) transplatin, D) carboplatin, E) oxaliplatin. The stoichiometry of each platinum/protein adduct is 3:1.

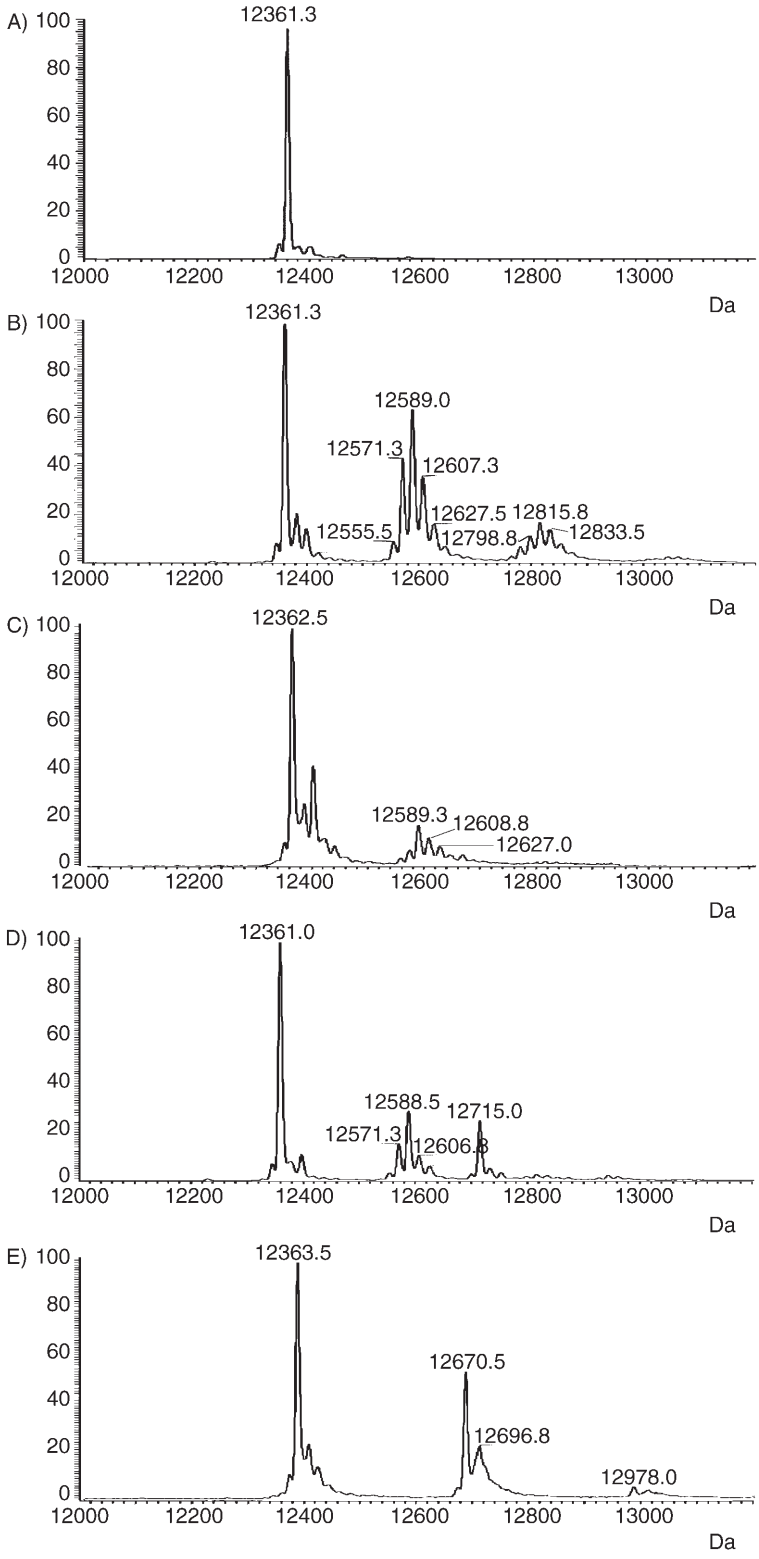


Figure 2. Deconvoluted ESI-MS spectra of A) cytochrome c and its platinum adducts: B) cisplatin, C) transplatin, D) carboplatin, E) oxaliplatin. The stoichiometry of each platinum/protein adduct is 3:1.

and 3:1 platinum–protein adducts are detected under the applied solution conditions (72 h incubation, 3:1 Pt/protein ratio).

Analysis of the ESI-MS spectrum obtained for the carboplatin derivative reveals a rather different situation (Figure 2D). Indeed, two clearly distinct adducts are observed, both manifesting a 1:1 platinum/protein stoichiometry, while adducts with a higher platinum content are not detected. In detail, we observe a first multiplet centred at 12589 Da, similar in shape to that found in the cisplatin derivative, and a second one, at 12715 Da of comparable intensity. The latter most likely corresponds to addition of a $[Pt(NH_3)CBD]$ (CBD = *cis*-(1,1-cyclobutanedicarboxylato)) fragment to the protein. Failure to observe the peak of a doubly platinated protein adduct suggests that binding of the two distinct platinum fragments to cyt c is competitive for the same site (most likely Met65; see below). Notably, for longer incubation times, the peak at 12715 becomes much weaker while the peak at 12589 Da increases its intensity; this suggests progressive release of the *cis*-(1,1-cyclobutanedicarboxylato) ligand from the protein-bound platinum centre.

Finally, the ESI-MS spectrum of the oxaliplatin derivative is shown in Figure 2E. An intense peak is observed at 12670 Da that might well correspond to the binding of a $[Pt(R{=}(NH_2)_2)]^{2+}$ (R = cyclohexane) moiety to cyt c. Moreover, the peak of a 2:1 adduct, in which two fragments of the above kind are associated to the protein, is clearly observed at 12977 Da.

Additional ESI-MS measurements were performed on Pt–cyt c adducts prepared under more drastic conditions (Pt/cyt c ratio of 10:1; 168 h incubation time, 37 °C). In all cases these conditions led to formation of adducts with greater Pt/cyt c stoichiometries and to substantial decreases in the peaks of the native protein and of the monoplatinated adduct. A representative example is shown in Figure 3. The ESI-MS spectrum of a cyt c sample incubated for one week with a tenfold molar excess of cisplatin is dominated by three peaks of comparable intensity corresponding to the 2:1, 3:1 and 4:1 Pt–cyt c adducts. Remarkably, a very weak peak corresponding to the free protein can only just be observed at 12361.8 Da; this suggests that almost complete protein platination has occurred.

Overall, the results reported above provide quite detailed insight into the reactivity of the various platinum complexes with cyt c, as judged from the nature of the resulting products. It is shown that all tested platinum compounds, when presented to the protein in a 3:1 molar excess for 72 h at 37 °C generate a certain number of stable platinum–protein adducts, with a net predominance of the monoplatinated ones. Very similar cyt c platination levels were afforded following treatment with the four different platinum compounds under identical conditions. In addition, higher but still comparable platination levels were obtained for cyt c samples treated for one week with a tenfold molar

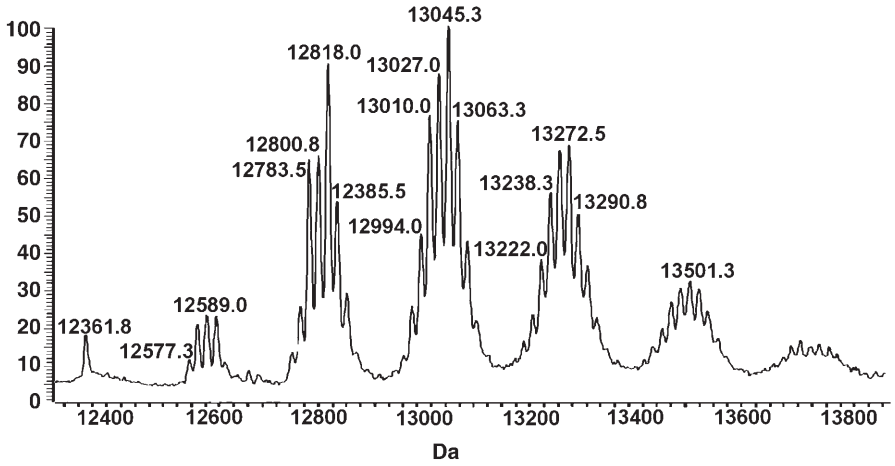


Figure 3. Deconvoluted ESI-MS spectra of the cisplatin adduct with cyt c in a 10:1 ratio, incubation time 168 h at 37 °C.

excess of either cisplatin or carboplatin. In fact, platination levels of 6.7 ± 0.5 and 5.0 ± 0.5 Pt moles per cyt c were found for cisplatin and carboplatin, respectively.

Thus, under the present experimental conditions, the four investigated platinum compounds turn out to exhibit a roughly similar pattern of reactivity with cyt c. This finding is of particular interest and novelty, being in striking contrast to current opinions concerning the comparative reactivity of the investigated platinum drugs. Indeed, the four platinum compounds selected for our study are known to exhibit greatly different stability patterns under physiological-like conditions.[15] For instance, carboplatin has been reported to hydrolyse about 100-fold less rapidly than cisplatin.[15] Equally, oxaliplatin has been shown to hydrolyse far more slowly than cisplatin.[15] The higher stabilities of carboplatin and oxaliplatin towards are generally reflected in a lower reactivity with DNA and with other proteins.[16] Thus it is really surprising that all the platinum compounds tested here were found to platinate cyt c at a substantially similar level. It can be inferred that cyt c plays some active role in enhancing the reactivity of the kinetically stable carboplatin and oxaliplatin.

The specific sites of protein platination still require unambiguous identification. It is known, from divalent platinum solution chemistry, that complexes of the "soft" platinum(II) ion display high affinity for sulfur-containing groups: they are expected to bind readily to cysteine and methionine sulfurs and, with lower affinity, to the imidazole nitrogens of histidines.[17] In cyt c, only Met65, His26 and His33 are freely available for platinum coordination. In line with the reported higher affinity of platinum(II) complexes for sulfur ligands, and previous spectroscopic results concerning the interaction of cisplatin with cyt c,[18] we propose that Met65 represents the primary binding site for the tested platinum drugs, while the imidazole groups of His26 and His33 offer secondary binding sites. The above analysis of the ESI-MS spectral features of the various platinum derivatives highlights predominant formation of monoadducts in which the platinum fragments are most likely coordinated to Met65. Interestingly, the two distinct monoplatinated adducts obtained upon reacting cyt c with carboplatin imply that both fragments compete for the strong Met65 "primary" site rather than binding to "secondary" His sites.

In conclusion, we have shown that classical platinum(II) drugs produce cyt c adducts sufficiently stable to survive the soft ionisation procedures of the ESI-MS experiment. The stoichiometry of the individual adducts and the nature of the protein-bound metal fragments have been fully characterised by interpreting their respective ESI-MS spectra. Remarkably, and surprisingly, all tested platinum(II) drugs were found to exhibit roughly comparable reactivity profiles towards this protein. We suggest that Met65 represents the primary anchoring site for platinum(II) compounds.

Beyond the specific information gained on platinated cyt c derivatives, the present study further exploits the ESI-MS technique as an election and direct method for the accurate molecular description of weak but biologically relevant metal–protein adducts. Valuable information on the comparative reactivity of families of metallodrugs towards protein targets can be straightforwardly derived from the application of the described experimental approach.

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Keywords: antitumor agents · cytochromes · ESI-MS · platinum · reaction mechanisms

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[14] Cisplatin, transplatin, carboplatin and oxaliplatin were purchased from Sigma–Aldrich. Horse heart cytochrome c was also from Sigma. Samples were prepared in ammonium carbonate buffer (25 mM, pH 7.4) with a protein concentration of 5×10^{-4} M, and platinum-to-protein ratios ranging from 3:1 to 10:1. The reaction mixtures were incubated for different time intervals (24 h, 72 h and 1 week) at 37 °C. Samples were extensively ultrafiltered by using Centricon YM-3 (Amicon Bioseparations, Millipore Corporation) in order to remove the unbound platinum complex. After the samples had been diluted 100-fold with MilliQ water, ESI-MS spectra were recorded by direct introduction at a flow rate of 3 μL min^{-1} in a LTQ linear ion trap (Thermo, San Jose, CA) equipped with a conventional ESI source. The specific conditions used for these experiments were as follows: spray voltage = 3.5 kV, capillary voltage = 40 V and the capillary temperature was kept at 353 K. The sheath gas was set at 13 (arbitrary units) whereas sweep gas and auxiliary gas were kept at 0 (arbitrary units). ESI spectra were acquired by using Xcalibur software (Thermo), and deconvolution was obtained by using Bioworks software (Thermo). The mass-step size in deconvolution calculation was 0.25 Da, and the spectrum range considered was 1100–2000 *m/z*. The same experiments were repeated at various capillary temperatures from 90 to 180 °C, but the peak patterns and relative abundances were not influenced (data not shown). ICP-OES spectra of the samples were recorded on an Optima 2000 instrumentation (Perkin–Elmer).

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