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Journal of Chromatography A, 719 (1996) 171–179

JOURNAL OF
CHROMATOGRAPHY A

Amino acid and amino sugar determination by derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate followed by high-performance liquid chromatography and fluorescence detection

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Abstract

A method for the derivatization of amino acids with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) for use with HPLC was tested. The reproducibility, linearity and limits of detection are similar to those obtained with other methods, such as the *o*-phthalaldehyde and 9-fluorenylmethoxycarbonyl methods. The ease of sample preparation and the possibility of amino sugar derivatization and resolution are the reasons why this method was chosen for simultaneous amino acid and amino sugar analysis. The need for a previous step of amino sugar reduction was studied and it was concluded that it is not necessary.

1. Introduction

In recent years, growing interest in the study of glycoproteins, proteoglycans, etc., in the biological and biochemical field has led to attempts to develop methods to determine their most important components, amino acids and amino sugars. As both can be released in a conventional acid hydrolysis, a simultaneous method of analysis would be useful.

Traditionally, the determination of amino acids was based on ion chromatography with post-column derivatization with ninhydrin and UV detection. However, the development of HPLC in the last 15 years has changed their form of analysis. Owing to their great polarity and the absence of chromophores in most of them, a derivatization prior to analysis by RP-HPLC is necessary. This led to the appearance of many derivatization reagents, e.g., PITC [1–4], Dabsyl-Cl [5], Dansyl–Cl [6–10], OPA [11–14] and FMOC-Cl [15–25].[1] These reagents can also derivatize amino sugars, because the amino group is responsible for the reaction. However, the analysis of these compounds presents an additional problem. In nature, amino sugars are

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[1] Abbreviations used: PITC, phenyl isothiocyanate; Dansyl-Cl, 1-dimethylaminonaphthalene-5-sulphonyl chloride; Dabsyl-Cl, 4-dimethylaminoazobenzenesulfonyl chloride; OPA, *o*-phthalaldehyde; FMOC-Cl, 9-fluorenylmethyl chloroformate; AQC, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate; NHS, N-hydroxysuccinimide; AMQ, 6-aminoquinoline; AABA, α-aminobutyric acid; N-Leu, L-norleucine.

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SSDI 0021-9673(95)00372-X

found in two anomeric forms in equilibrium and, in consequence, two peaks appear in the chromatogram. Many workers [2,3,13] have proposed a prior reduction as a solution, resulting in only one peak per hexosaminitol in the chromatogram.

An important aspect of the study of amino acid and amino sugar analysis is to establish the optimum derivatization. As the amount of sample is very high in these cases, the derivatization method should be simple and rapid. PITC derivatization, however, is long and involves several stages of drying under vacuum. Another disadvantage of this method is that UV detection is required and therefore the limits of detection are in the picomole range. Fluorescent derivatives that allow limits of detection at the femtomole level would be preferable. Although FMOC and OPA do not have these disadvantages, they have others. OPA does not derivatize secondary amino acids, such as Pro or Hpr. Moreover, the derivatives show low stability, of a few minutes. On the other hand, with FMOC the excess of derivatization reagent can give rise to problems if it is not removed by extraction with pentane [15,16,18,19,24] or if it is not derivatized with an amine whose derivative does not interfere [17,20–22, 25]. In addition, neither FMOC nor OPA allows a good chromatographic resolution of amino acids and amino sugars.

After earlier tests on the possible viability of some carbamates acting as derivatizing amino acid reagents [26,27], recently a new derivatization reagent, AQC [28–32], has appeared. It gives stable derivatives with a fluorescent response and its excess does not need to be removed as it does not interfere. Cohen and co-workers [30,31] applied this method to the determination of amino acids in different matrices. One of these is glycoproteins, which can be analysed for amino sugars and amino acids. However, amino sugars are not determined.

In this work, we studied the possibilities of the AccQ-Tag method, based on derivatization with AQC, for the simultaneous determination of amino acids and amino sugars and the need for a prior reduction stage.

2. Experimental

2.1. Reagents and materials

Sodium borohydride was supplied by Merck (Darmstadt, Germany). Sodium hydroxide and hydrochloric acid were purchased from Scharlau (Barcelona, Spain). Amino acid standard (containing 2.5 m*M* of seventeen proteic amino acids) was obtained from Pierce (Rockford, IL, USA). Glucosamine and galactosamine, the amino sugars employed, and L-norleucine, used as an internal standard, were purchased in pure form from Merck, and α-aminobutyric acid was provided by the Organic Chemistry Department of the Institut Químic de Sarrià (Barcelona, Spain). Borate buffer and AQC, as a powder, and acetonitrile to reconstitute AQC were obtained from Waters (Milford, MA, USA). Sodium acetate and phosphoric acid were supplied by Panreac (Barcelona, Spain) and triethylamine by Merck. Acetonitrile of HPLC grade was obtained from SDS (Peypin, France). Deionized water was produced with a Millipore Milli-Q system from Waters. Chitin from crab shells was supplied by Sigma (St. Louis, MO, USA), soya protein by the Organic Chemistry Department of the Institut Químic de Sarrià and sewage sludge by DARGISA (Girona, Spain).

2.2. Hydrolysis

A 2.5-g amount of sample was hydrolysed with 90 ml of boiling 6 *M* HCl for 6 h. The hydrolysed sample was filtered and diluted to 100 ml. A 100-μl volume of internal standard was added to 5 ml of the hydrolysed sample.

2.3. Reduction

A 10-μl volume of the standard or hydrolysed sample was evaporated to dryness in a vacuum oil system and 25 μl of 2% sodium borohydride in sodium hydroxide were added. After a minimum of 2 h of reduction at room temperature, the borohydride was discharged with 50 μl of 0.5 *M* HCl.

2.4. Derivatization

A 10-μl volume of the hydrolysed sample (with the internal standard added) was evaporated to dryness in a vacuum oil system and reconstituted with 20 μl of 20 m*M* HCl, then 60 μl of 0.2 *M* borate buffer (pH 8.8) were added. The derivatives were formed with 20 μl of AQC in acetonitrile and heated for 5 min at 40°C to accelerate the conversion of di-Tyr into the mono-Tyr derivative.

2.5. Chromatographic instruments and conditions

The HPLC system was a Waters Model 600E pump with a Rheodyne (Cotati, CA, USA) 5-μl loop injector, an AccQ-Tag C_{18} (4 μm) column (150 mm × 3.9 mm I.D.), a column heater and a Model 470 fluorescence detector, all from Waters. Millennium Software, from Waters, was used to control the system and to collect and integrate data.

A ternary gradient system was used. Mobile phase A consisted of 140 m*M* sodium acetate–17 m*M* TEA titrated to pH 5.05 with phosphoric acid. Mobile phase B was water and mobile phase C was acetonitrile. The gradient conditions are given in Table 1. The AccQ-Tag column was thermostated at 37°C and operated at a flow-rate of 1.0 ml/min.

The fluorescence excitation and emission wavelengths were set at 250 and 395 nm, respectively.

Table 1
Gradient conditions

Time	Acetate buffer (%)	Water (%)	Acetonitrile (%)
0	100	0	0
0.5	99	0	1
18	95	0	5
19	91	0	9
29.5	83	0	17
33	0	40	60
55	100	0	0

3. Results

3.1. Reduction

Standards of amino acids and amino sugars with and without reduction were derivatized and chromatographed (Fig. 1).

The reduced standard provided a chromatogram in which there was only one peak per amino sugar. In the chromatogram of the non-reduced standard there were two peaks per amino sugar, which were repetitive, and none of them interfered with those of amino acids. In addition, the comparison between the first peak of the hexosamine and the peak of the hexosaminitol revealed only a small decrease in sensitivity.

3.2. Analytical reproducibility

Peak-area repeatibility, obtained from six injections of standard on the same day, ranged from 5.7 to 9.9% relative standard deviation (R.S.D.). The reproducibility, from five injections on several days, was slightly higher (R.S.D. 6.1–11.6%). The peak-height precision was slightly poorer (data not shown). For the internal standard these values were higher: the repeatibility was 6.9% and the reproducibility was 34.2% (R.S.D.).

3.3. Linearity and limits of detection

Analyses of serial dilutions of the standard mixture, from 0.1 to 13 μ*M*, showed a linear response in this range with $r > 0.99$ (Table 2). Limits of detection ranged from 49 to 780 fmol.

3.4. Derivative stability

The derivatives showed high stability. The signal was almost at 100% until 7 days after

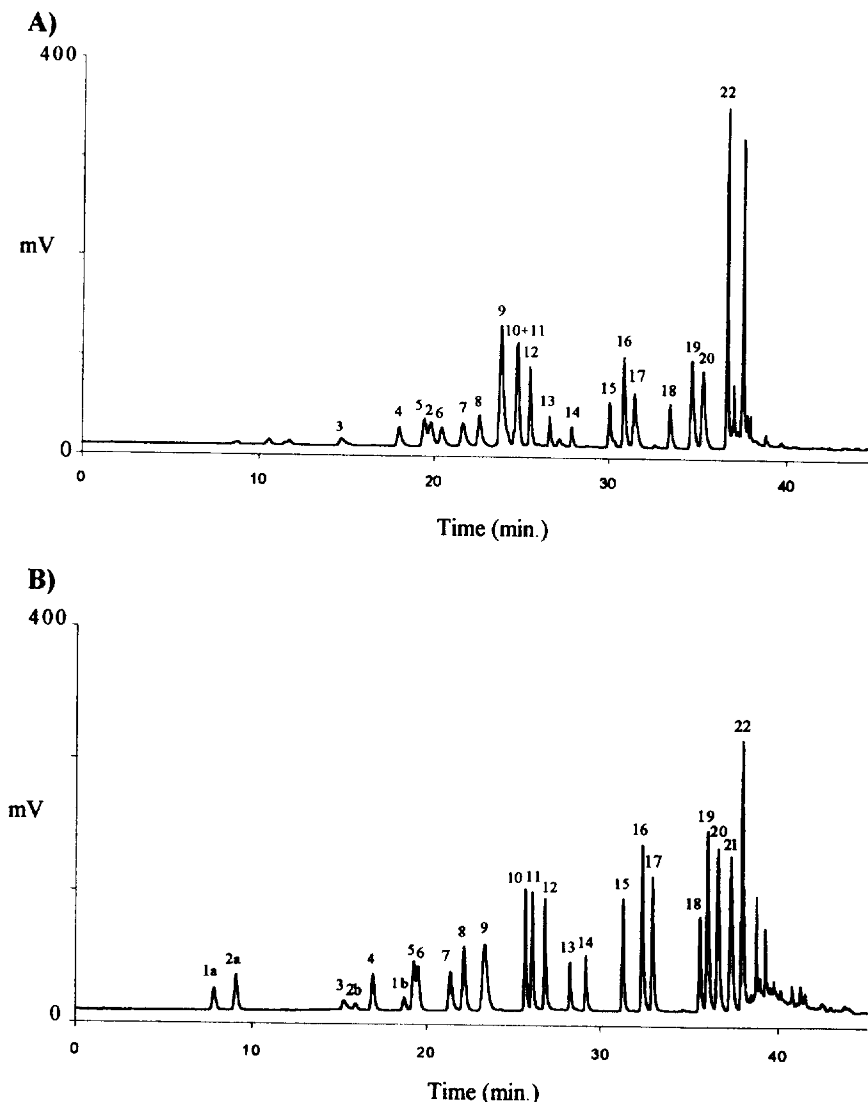

Fig. 1. Chromatograms of (A) a reduced standard and (B) a non-reduced standard. Peaks: 1a = β-GalN; 1b = α-GalN; 2a = β-GlcN; 2b = α-GlcN; 2 = GlcN-ol; 3 = AMQ; 4 = Asp; 5 = Ser; 6 = Glu; 7 = Gly; 8 = His; 9 = NH_3; 10 = Arg; 11 = Thr; 12 = Ala; 13 = Pro; 14 = AABA; 15 = Tyr; 16 = Val; 17 = Met; 18 = Lys; 19 = Ile; 20 = Leu; 21 = N-Leu; 22 = Phe.

derivatization and the decrease was not important after 27 days (Fig. 2).

3.5. Internal standard

Quantification was effected with the internal standard method, as it is usual in amino acid determination, using the AccQ-Tag method with α-aminobutyric acid (AABA) as internal standard. However, this amino acid shows poorly reproducible behaviour, making it difficult to quantify. An exhaustive study, in which AABA and N-Leu were added to samples in different media (HCl, trifluoroacetic acid, phosphate buffer, citrate buffer, etc.) and with different amino acid concentrations, showed that the first amino

Table 2
Linear response from 0.1 to 13 μM (area = $A + B \cdot$ concn.)

Compound	$A \times 10^{-4}$	$B \times 10^{-9}$	r
GlcN	4.30	4.98	0.9917
Asp	9.32	3.88	0.9929
Ser	4.25	6.55	0.9997
Glu	0.17	3.91	0.9924
Gly	2.81	6.44	0.9937
His	0.79	6.04	0.9999
Arg	2.91	14.37	0.9961
Thr	2.91	14.37	0.9961
Ala	1.66	7.96	0.9790
Pro	1.29	2.91	1.0000
AABA	−0.14	4.47	1.0000
Tyr	0.87	7.32	0.9901
Val	2.90	11.94	0.9968
Met	0.34	36.23	0.9980
Lys	−0.05	6.32	0.9990
Ile	1.03	12.82	0.9974
Leu	0.92	37.80	0.9987
Phe	4.70	24.14	0.9985

acid had great variability whereas the second gave very reproducible results. Therefore, N-Leu was adopted as the internal standard for quantification.

3.6. Applications

This method was applied to the analysis of three very different samples: soya protein, chitin and sewage sludge-amended soils, all of them hydrolysed in 6 M HCl for 6 h. The corresponding chromatograms are shown in Fig. 3. The quantification results are given in Table 3.

Soya protein is a typical food sample and only releases amino acids. In contrast, chitin is an N-acetylglucosamine polymer and only the peaks of this compound, ammonium and the internal standard appeared in the chromatogram. Sewage sludge-amended soil is a very complex matrix in

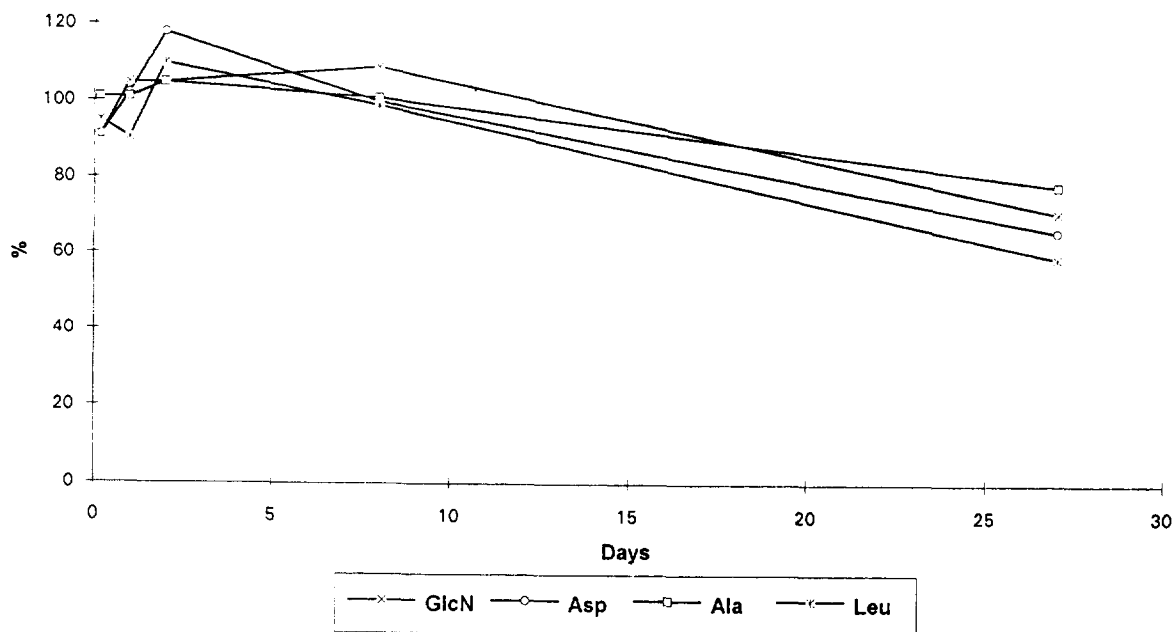

Fig. 2. Plot of decrease in derivative stability versus days after derivatization. × = GlcN; ○ = Asp; □ = Ala; * = Leu.

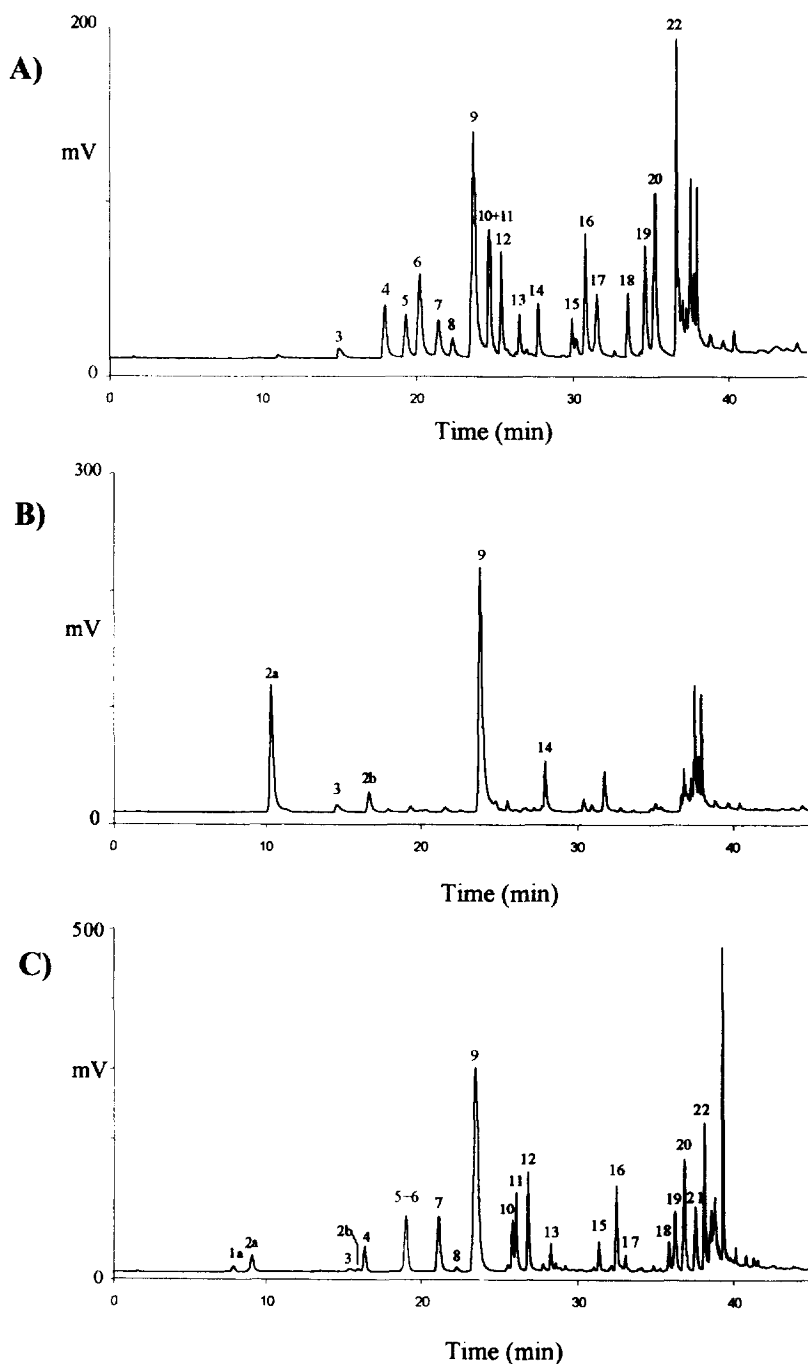

Fig. 3. Chromatograms of (A) hydrolysed soya protein, (B) hydrolysed chitin and (C) hydrolysed sewage sludge. Peaks: 1a = β-GalN; 1b = α-GalN; 2a = β-GlcN; 2b = α-GlcN; 2 = GlcN-ol; 3 = AMQ; 4 = Asp; 5 = Ser; 6 = Glu; 7 = Gly; 8 = His; 9 = NH_3; 10 = Arg; 11 = Thr; 12 = Ala; 13 = Pro; 14 = AABA; 15 = Tyr; 16 = Val; 17 = Met; 18 = Lys; 19 = Ile; 20 = Leu; 21 = N-Leu; 22 = Phe.

Table 3
Results for analysis of real samples

Compound	Sample		
	Soya protein (g compound/kg sample)	Chitin (g compound/kg sample)	Soil (mg compound/kg sample)
GalN	–	–	744.5
GlcN	–	647.1	1741.2
Asp	91.6	–	1155.1
Ser	34.8	–	}1734.5
Glu	177.7	–	
Gly	35.3	–	864.2
His	18.8	–	87.2
Arg	}103.1	–	670.4
Thr		–	773.4
Ala	36.8	–	814.4
Pro	48.4	–	584.2
Tyr	27.6	–	384.9
Val	29.7	–	558.5
Met	32.6	–	175.4
Lys	51	–	450.1
Ile	28.1	–	468.6
Leu	59.1	–	875.6
Phe	51.4	–	680.7
Total	826	647.1	12762.9

which there are both types of compounds, which were perfectly resolved.

4. Discussion

The simultaneous determination of amino acids and amino sugars has been studied by some workers [3,13], their methods being the traditional ones of RP-HPLC with precolumn derivatization, but they have the typical advantages and disadvantages and they usually need a previous step of amino sugar reduction. Here, the AccQ-Tag method is proposed because of the ease of sample preparation, stability of the derivatives and, mainly, the good chromatographic resolution.

In contrast to the OPA method, which needs combination with other methods [12] to effect the determination of secondary amino acids, the peak of the Pro derivative, one of the most important secondary amino acids, appears in the chromatogram after a single derivatization with AQC (Fig. 1). It should also be taken into account that an excess of derivatizing reagent should not interfere. AQC reacts with water to release two by-products. One of them (NHS) is not fluorescent and, although the other one (AMQ) is, as it appears in a zone of the chromatogram without peaks and with a low signal, it does not interfere.

Because of the good resolution of the method, not only the peaks of reduced amino sugars can be resolved from those of amino acids, but also those of non-reduced amino sugars. As reduction makes the analysis last a minimum of 2 h longer than usual and it needs an additional step of vacuum drying, the only fact that would justify its use would be a very important gain in sensitivity. However, the chromatograms show that the difference between the hexosamine peak and the corresponding hexaminitol peak is a minimum, so reduction is unnecessary.

The reproducibility and repeatibility are simi-

lar to those of other RP-HPLC methods of amino acid analysis. Peak area was chosen for quantification as it is more reproducible than peak height.

In contrast to other methods, such as with PITC, and because of the fluorescence detection, this method has limits of detection in the femtomole range for both amino acids and amino sugars.

The stability of the AQC derivatives is very high. In contrast to other methods, such as with OPA, whose derivatives are stable for only a few minutes, and FMOC-Cl, which maintain the response for several hours, AQC derivatives are stable for 1 week and do not show a decrease in signal until nearly 3 weeks after derivatization. This is of great practical importance. In order to use an automatic injector when OPA is the chosen method, it must necessarily derivatize in the injector. The AQC method allows automated injection without the need for derivatization in the injector. A large number of samples can be derivatized outside the injector without the risk of a decrease in the signal.

The matrices tested demonstrate that the method is suitable for the analysis of very different samples. A simple sample, such as chitin, was analysed with only a peak from glucosamine. Further, a sample with a very complex matrix, sewage sludge-amended soil, was also analysed with excellent results for all amino acids and amino sugars.

5. Conclusions

The method based in a derivatization with AQC, chromatographic separation by RP-HPLC and fluorescent detection allows the resolution of amino acids and amino sugars with repeatibility, reproducibility and linearity similar to those of other methods and with good limits of detection. Derivatization is rapid and easy and prior reduction of amino sugars is unnecessary. As a result, very stable derivatives are obtained. Hence the AQC method is suitable for the simultaneous determination of amino acids and amino sugars.

Acknowledgements

The authors thank the CICYT (Spain) (Reference: NAT 91-0340), for financial support and J.Ll.Ll gratefully acknowledges the support of DGICYT (Spain).

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