Application of Curve Fitting in Thin-Layer Chromatography–Flame Ionization Detection Analysis of the Carbohydrate Fraction in Marine Mucilage and Marine Snow Samples from Italian Seas

Mauro Mecozzi[1,*] and **Zsuzsa Pápai**[2]

[1]Central Institute for the Scientific Marine Research, Via di Casalotti 300, 00166 Rome, Italy and [2]Department of Analytical Chemistry, University of Veszprém, P.O. Box 158, Veszprém, H-8201 Hungary

Abstract

This paper presents a thin-layer chromatographic–flame ionization detection (TLC–FID) procedure to characterize the carbohydrate fraction of marine mucilage and marine snow samples from the Italian Seas. The identification of the different carbohydrate subfractions is supported by the application of a deconvolution procedure based on a new mathematical function for describing chromatographic peaks and enhancing their resolution. The joint- approach TLC–FID analysis and deconvolution procedure allows for the characterization of the carbohydrate fraction of the marine samples in a single step without using the different derivatization procedures requested by the most common gas chromatography and high-performance liquid chromatographic methods for carbohydrate analysis. In fact, the results obtained by the TLC–FID procedure show that different neutral, uronic acid, and aminosugar subfractions can be present simultaneously in these samples. Moreover, the results support some hypotheses about the causes of the presence of mucilages in the Italian Seas.

Introduction

Several aggregates of organic matter, suspended and floating along the water column, are often present during spring and summer seasons in the Italian Seas. These aggregates are different for dimension and form; the aggregates with dimension lower than 1 cm are called marine snow, and the aggregates having different forms such as flocs, strings, clouds, and scums with dimensions varying from some centimeters to several hundred meters are called mucilage (1,2) (Figure 1 shows an example of these aggregates). In the mucilage events of these last years observed in the Adriatic and Tyrrhenian Seas, this phenomenon caused serious damage to tourism and fishery industries, and, as a consequence, several studies have been supported to clarify the causes of the mucilage presence. Some studies have shown that carbohydrates are a relevant fraction of the chemical composition of mucilages (3), which are fundamentally exudates of marine phytoplankton (4), but real comprehension of the pathways involved in their formation requires the elucidation of their chemical structure. With this aim, in the previous years a colorimetric method was developed to analyze total carbohydrates (5) and a thin-layer chromatographic (TLC)–flame ionization detection (FID) method to analyze the lipid fractions of these samples (6).

In particular, some studies have shown that the carbohydrate fraction has a fundamental role in the aggregation characteristics of the marine organic matter (7,8). The full characterization of this fraction and the comprehension of the possible pathways of their formation are of obvious importance. For the determinations of monosaccharides in environmental samples, gas chromatographic (GC) and high-performance liquid chromatographic (HPLC) methods are often preferred to TLC methods, although the scientific literature reports interesting TLC procedures to analyze carbohydrates in different matrices. Cellulose layers are reported for analyzing carbohydrates (9), yet some applications of silica gel layers are reported in food analysis (10). Whatman K6 plates were used for carbohydrates analysis by derivatization as alditols and alkaline $AgNO_3$ dipping detection (11) and methylation with densitometric detection (12).

Several high-performance thin-layer chromatographic (HPTLC) methods have also been reported. HPTLC-quality silica gels and *N*-(1-napthyl)ethylenediamine-sulfuric acid reagent have been proposed for determining the sugar composition of food gum polysaccharides (13); amino-bonded HPTLC plates have been used for the analysis of monosaccharide mixtures (14) and fructoolisaccharides (15). The use of TLC–FID for carbohydrate analysis is not very common because it is generally restricted to the analysis of samples with a simple sugar composition (16) and to the study of the products of enzymatic hydrolysis of chi-

* Author to whom corresondence should be addressed: email mecoma@rdn.it.

toolisaccahrides (17). This depends mainly on the chromatographic behavior of most sugars whose polar hydroxyl groups have strong adsorption bonds with the silica gel of Chromarods and the large number of positional isomers that produce individual broad bands and consequent overlapping peaks (16). However a TLC–FID approach for carbohydrate analysis can be an alternative procedure to the GC analysis of neutral and uronic carbohydrates (18) and aminosugars (19), which are methods requiring the chemical derivatization of sugars.

This paper describes a TLC–FID procedure to identify the contribution of the neutral, uronic acid, and amino sugar subfractions present in marine snow and mucilage samples of Italian Seas.

Experimental

Sampling

Mucilage samples were taken from the Tyrrhenian Sea in different places. Some mucilages were sampled along the coasts of Elba Island (Central Italy) between 1999 and 2001 and the coasts of Giglio Island (Central Italy) in summer 1999. Other mucilages were sampled along the coasts of the countries Circeo, Scario, and Procida (South Italy) on October 2000. Marine snow samples, Porto Turistico 02, Riovivo 02, and Casaccia 02 were collected along the coasts of Elba Island (Tyrrhenian Sea, Central Italy) in May 2002. All of the samples were stored frozen at –20°C until beginning the chemical analysis.

Figure 1. Mucilage aggregates observed in Adriatic Sea in summer 1997. The length of each sample was approximately 15 cm.

Mucilage pretreatment

All the samples were dialyzed by a Spectrum 1000 dialysis membrane (Houston, TX) and lyophilized prior to any analysis. Samples were stored in dark glass containers.

Equipment

Planar chromatographic analysis was performed using a TLC Iatroscan MARK V equipped with a flame ionization detector (NTS, Rome, Italy) and connected to a PC for the acquisition of chromatograms as ASCII files.

Colorimetric measurements were performed using a PerkinElmer Lambda 40 UV–vis spectrophotometer (PerkinElmer, Norwalk, CT) connected to a PC for the acquisition of chromatograms as ASCII files.

TLC–FID analysis of marine mucilage and snow samples

Each lyophilized sample (100–300 mg) was added to 5.0 mL 1M HCl and placed in an oven bath at 100°C for 2.5–3 h. After cooling, the supernatant was separated by centrifugation. The acid solution (1 µL) was spotted at the origin of the Chromarod S II silica gel 5 µm, using a microliter syringe. The development of the rod to resolve the qualitative carbohydrate fraction was performad by an elution phase consisting of acetonitrile–ethyl acetate–isopropyl alcohol at 82.5:10:7.5 (v/v), respectively. When the elution phase covered the 70% of the total length of the rod, it was dried in an oven at 65°C. The development was repeated three times. The constant humidity was reached by placing a saturated NaCl solution in the elution tank (16).

Standard solutions containing neutral uronic and amino sugars simultaneously and including also myoinositol as internal standard were used for the identification and estimation of the different carbohydrate subfraction and for the estimation of total carbohydrate amount. The differences between the R_f value of the internal standard and the R_f value of the peaks was used for the identification of the monosaccharides. Regression curves having correlation coefficients within 0.992 and 0.997 were obtained for all of the monosaccharides tested. By means of the described procedure and operating on a 300-mg sample, a detection limit ranging between 2.5% and 3% (w/w) can be obtained for the analysis of total carbohydrate amount.

Application of the fitting procedure to the TLC–FID chromatograms for the identification of neutral uronic acid and amino sugars subfractions

As most TLC–FID chromatograms show overlapped peaks, a deconvolution procedure is necessary to enhance the peak resolution in order to improve the identification and the quantitative estimation of carbohydrates. The deconvolution of chromatographic peaks is based on the use of mathematical functions (i.e., Gaussian, log-normal, Gamma, Weibull, and modified Gaussian functions), which describe the peak shape (20). One of the most used functions is the exponentially modified Gaussian function (EMG), which is the result of the convolution of a Gaussian function and an exponential decay function:

$$y(t) = h(t)*f(t) \qquad \text{Eq. 1}$$

where y(t) is the convoluted function describing the chromatographic peak, h(t) is the function describing a pure Gaussian peak, and f(t) is the exponential decay function:

$$h(t) = h_{max} \exp\{-(t - t_R)^2/2\sigma^2\} \quad \text{Eq. 2}$$

$$f(t) = \Gamma^{-1} \exp\{-t/\Gamma\} \quad \text{Eq. 3}$$

where h_{max} is the maximum peak height, t_R is the retention time, t is the time, σ is the standard deviation, and Γ is a constant that quantitates the decay time of the system.

The EMG model has been submitted in different studies in order to improve its efficiency in describing chromatographic peaks; Torres-Lapasió (21) has proposed a mathematical function of the EMG peaks in which the standard deviation of a pure Gaussian peak is replaced by the use of a polynomial function according to:

$$h(t) = h_{max} \exp -(1/2)*([t - t_R] / [\sigma_0 + \sigma_1(t - t_R) + \sigma_2 (t - t_R)^2 + \ldots\ldots])^2 \quad \text{Eq. 4}$$

and showing that the results obtained by the proposed models are even better than those obtained by the EMG model in resolving binary and ternary mixtures of compounds having overlapped peaks. Economou et al. (22) have applied fast Fourier and Hartley transforms for the deconvolution of EMG peaks showing the aspects of resolution enhancement, linearity, background correction, and data distortion on Gaussian peaks.

However, in spite of the wide use of the EMG model, it also has some disadvantages related to the estimation of σ and Γ because their accuracy is influenced by the asymmetry of the peaks (20,23), and new deconvolution procedures have been proposed to solve the problems arising from the use of the EMG model.

As far as the use of deconvolution procedure in planar chromatography, it is necessary to specify that TLC peaks have to be described by binomial distributions, whereas GC and HPLC peaks are Gaussian-type shape. However, there is usually a negligible differences between models (24) so that the deconvolution procedures applied in gas and liquid chromatography can be applied to TLC peaks as well. Here we used a deconvolution procedure presented in a previous work (23) to improve the identification of the carbohydrate subfractions present in the aggregate samples of marine organic matter such as mucilages and marine snow. This procedure is based on studies related to the possibility of describing chromatographic peaks by mathematical function, and this approach has become a universal method for the characterization of peak shapes. Using curve fitting, the evaluation of chromatographic signals can be achieved successfully. In this case it is important to find the best mathematical function that corresponds perfectly to the digitized signal. An important application of the curve fitting represents the resolution of overlapped peaks; and in this work, a mathematical function is applied auspiciously for curve fitting, for which the function is presented for describing symmetrical and asymmetrical (tailing) peaks. The function contains four parameters, and its mathematical form in R_f domain is as follows:

$$f(R_f) = \begin{cases} 0, & \text{if } R_f < M - \dfrac{D(4 - a^2)}{2a} \\ H.\exp\left\{\left(\dfrac{4}{a^2} - 1\right) \cdot \left[\ln\left(1 + \dfrac{2a(R_f - M)}{D(4 - a^2)}\right) - \dfrac{2a(R_f - M)}{D(4 - a^2)}\right]\right\} \end{cases} \quad \text{Eq. 5}$$

where M is the location of the peak maximum, H is the peak height ($H > 0$), a is an asymmetry factor ($0 < a < 2$), and D is the standard deviation ($D > 0$). For curve fitting, the least-squares method was used. It is necessary to know the initial values of the parameters as preliminary information for the accurate fitting of the single and overlapped peaks. In case of symmetrical peaks, it is sufficient to know the peak maximum positions and the approximate values of the peak heights from the digital chromatogram and to start from these values. If the peak shape is asymmetrical, the previous information means the relatively exact knowledge of some other parameters (a, D). In case of overlapped peaks, the number of the parameters increases as a function of the number of fitted peaks. The fitted curve is suitable for a quick acquisition of chromatographic information, noise filtering, and baseline correction. The last operation can be carried out by adding a linear or quadratic term to equation 5. The nonlinear least-squares fitting was executed by the Gnuplot software (MS-Windows 32 bit version 3.7) (University of Northern Iowa, Cedar Falls, IA), which is fast (5–10 s/fit) and available on the Internet (http://www.gnuplot.info). The peak areas were calculated using MATLAB (MathWorks, Natlick, MA) version 5.2. and Pascal programs. The accuracy of the procedure can be easily tested by comparing the peak shape of deconvolved peak with the corresponding peak of the standard solution.

Colorimetric determination of total carbohydrate amounts

The colorimetric determination of total carbohydrates (25) was performed with the aim to validate the TLC–FID procedure for some samples, which showed a negligible amount of carbohydrates. The mucilage samples were hydrolyzed by adding 25 mL of 1M HCl to 20.0 ± 0.1 mg of lyophilized mucilage and placed in a water bath for 2.5–3 h at 100°C. After cooling and centrifugation, 2.0 mL of the acid solution was added to 2.0 mL of 5% (w/w) water solution of phenol and 10 mL of concentrated sulphuric acid in a glass tube. The tube was allowed to stand for 2 h at least in a shaker bath at 25–30°C.

Standard aqueous solutions of glucose were prepared in the range of 0–30 mg/L. The spectra were collected in the range of 525–450 nm in a 5-cm path length cell against a spectrophotometric blank consisting of 2.0 mL of water, 2.0 mL of 5% phenol, and 10 mL of concentrated sulphuric acid.

Chemical reagents

All of the reagents used for chemical analysis were of analytical-reagent grade Carlo Erba (Milan, Italy), and only ultrapure MilliQ water was used.

Results and Discussion

TLC–FID analysis of carbohydrates

As far as the chemical composition of the elution system is concerned, several mixtures were used, and better separation and reproducibility were obtained with the conditions reported in the Experimental section. Table I reports the relative R_f values for some selected carbohydrates; neutral carbohydrates were selected because they were already observed in a qualitative study of the mucilage composition (26), yet the presence of free uronic acids was already shown by a specific colorimetric analysis (3). Vice versa, no information is available about the presence of aminosugars, which can be also present in marine samples (27). The results of Table I show very close values of R_f for many carbohydrates so that environmental samples including all of the three types of carbohydrates could result in complex TLC–FID chromatograms with strong overlapped peaks. Figure 2 (reporting the chromatogram of a standard solution of neutral uronic and aminosugar carbohydrates) and Figure 3 (reporting the chromatogram of a real marine mucilage sample) confirm the complex behaviors of the TLC–FID chromatograms of environmental samples. The presence of strong overlapped peaks does not allow for a good estimation of the subfractions of neutral carbohydrates, uronic acids, and amino sugars, and a deconvolution procedure is necessary to enhance the peak resolution. Very satisfying results were obtained by applying the deconvolution procedure described previously. Some examples of the application of this procedure are shown in Figures 4 (i.e., the same chromatogram of Figure 3) and 5, and the results of the chemical speciation of carbohydrates, after the application of the fitting on all the chromatograms, are reported in Table II. The results of Figures 4 and 5 show that the qualitative identification of carbohydrates and their quantitative estimation can result quite difficultly because of the overlapped peaks. Vice versa, after the application of the fitting procedure, the identification of the carbohydrates present and their quantitative estimation are enhanced.

Table I. R_f Values of Some Monosaccharides Obtained by the Triple Elution and Acetonitrile Ethyl Acetate and Isopropyl Alcohol (82.5:10:7.5, v/v) System

Monosaccharide	R_f (cm)
Glucose	4.26
Galactose	4.26
Mannose	3.81
Arabinose	3.51
Xilose	3.56
Fucose	4.14
Ramnose	3.75
Glucuronic acid	3.46
Galacturonic acid	3.38
Glucosamine	4.81
Galactosamine	4.88

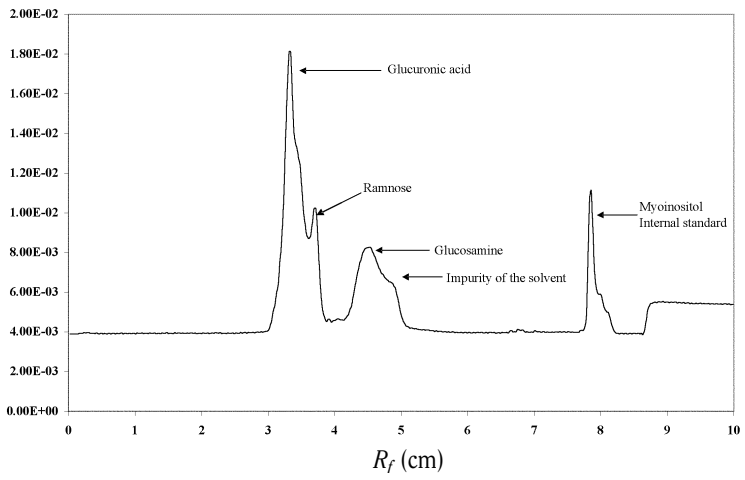

Figure 2. Chromatogram of a standard solution containing glucuronic acid (R_f = 3.4), ramnose (R_f = 3.7), glucosamine (R_f = 4.8) and myoinositol (R_f = 8) as internal standard.

At last it should be noted that the TLC–FID analysis is an excellent alternative method to colorimetric determination of the total carbohydrate amount in environmental samples. In fact, we compared the amount of total carbohydrates determined by measuring the total area in TLC–FID analysis and the amount determined by the colorimetric technique (25), obtaining a correlation coefficient of 0.983 at 95% of the statistical significance between the two methods (Table III).

Practical advantages of the application of TLC–FID analysis with the proposed curve fitting procedure

Though many mathematical functions are available for peak deconvolution in chromatography, it is quite difficult to identify the most powerful function because any function has some disadvantages that can reduce the efficiency of peak reconstruction. As far as the EMG function is concerned (maybe the most widely used to describe chromatographic peaks), detailed studies have shown that some peaks cannot be described by this function because there are differences between the shape of the deconvolved peak and the shape of the pure peak observed in the chromatogram of the standard solution (21,22). These observed differences are strictly related to the asymmetry sometimes present in the chromatographic peaks, which determines a non-Gaussian shape of the peak. The use of a polynomial function to describe the values of σ in an EMG distribution, as reported in equation 4, can improve but not solve all of the problems related to the deconvolution of asymmetric peaks because this type of function exhibits the defect that

$h(t \rightarrow \infty)$ 0, causing a problem of the baseline (22). Problems of modified peak shapes and baseline are not present in the use of a curve fitting procedure such as equation 5. These peculiar characteristics of the curve fitting procedure depend on the factor "a", which takes into account the asymmetry of the peak and on the characteristics of the intensity of the analytical signal "h", which tends to 0 when the retention time (i.e., the R_f value in TLC) tends to ∞.

Ecological evaluations on mucilage phenomenon according to the TLC–FID analysis

The TLC–FID analysis showed that mucilages can have a heterogeneous composition of carbohydrates because neutral carbohydrates, uronic acids, and amino sugars can be present. This evidence, never pointed out in previous studies, confirms the hypothesis that marine snow and mucilage are aggregates of organic matter produced according to the typical humification processes of the marine environment (3), in which all of the classes of organic compounds present in the marine environment contribute to the synthesis of the humic substance (27). In fact, it is relevant that the TLC–FID analysis shows the presence of refractory organic compounds such as uronic acids and amino sugars. As far as these carbohydrates are concerned, their role in the formation of the structural polymers of nonliving biomass is well recognized because it depends on the peculiar interaction of uronic and amino sugars with the cations of the aquatic environment such as Ca and Mg (28,29). In addition, the TLC–FID method provides also information on the ageing of mucilages because the low amount of carbohydrates observed in some samples (Table II), also confirmed by the results of the colorimetric analysis, depends reasonably on an advanced state of oxidative degradation of the organic matter in the exudates of marine phytoplankton (30).

Conclusion

The described TLC–FID procedure allowed for the analysis of the carbohydrate content of marine mucilage samples coming from the Italian Seas. The most relevant advantage obtained by this procedure is the general simplicity because with a single, inexpensive analysis it is possible to obtain information about the presence or absence of three different subfractions of carbohydrates such as neutral, uronic, and amino sugars, whereas the most used GC and HPLC methods require separate procedures with specific derivatisations to obtain the same information. Because of their specific separation ability, GC, HPLC, and also HPTLC methods obviously remain more powerful than TLC–FID for the identification of all the monosaccharides. However, TLC–FID analysis supported by the the application of an opportune curve fitting procedure is shown to be a good screening method for the preliminary analysis of carbohydrates in complex samples such as marine snow and mucilage.

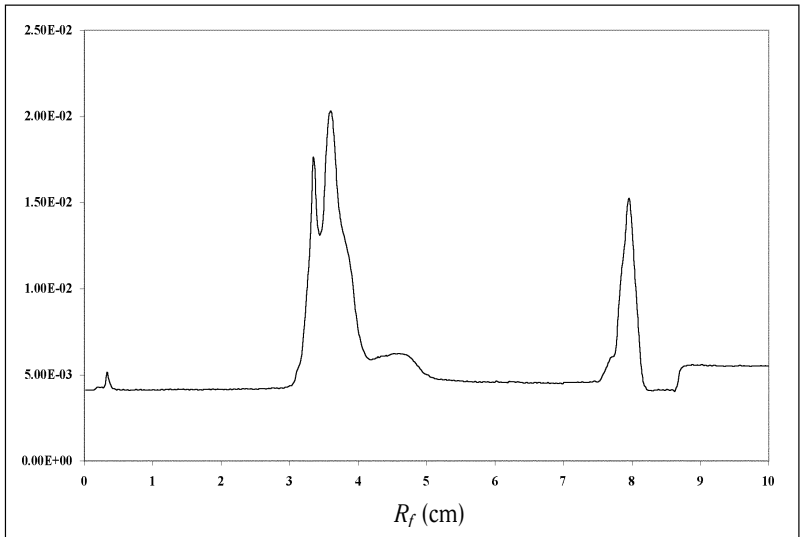

Figure 3. TLC–FID chromatogram of a mucilage sample (Circeo prof 00). The peaks with a R_f value ranging between 3 and 5 correspond to the identified carbohydrates, and the peak with a R_f value 8 is the internal standard.

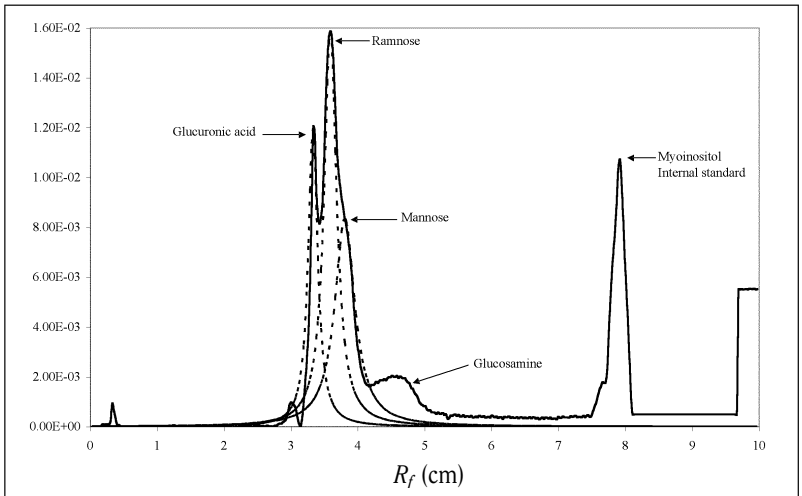

Figure 4. The result of the deconvolution procedure applied to the same chromatogram of Figure 3. The dotted lines show the reconstruction of each peak. R_f values are centimeters.

Table II. Concentration of the Principal Monosaccharides* Identified in the Mucilage Samples after the Fitting Procedure†

Sample	Glu	Gal	Ram	Fuc	Man	Ara	Aglu	Glm	TN	TU	TAS
Circeo prof 00	nd	nd	30	nd	25	nd	40	9	55	40	9
Riovivo 02	nd	nd	nd	nd	nd	nd	100	nd	nd	100	nd
Casaccia 02	nd	nd	nd	nd	nd	nd	100	nd	nd	100	nd
Circeo-3m 00	39	nd	nd	28	nd	3	nd	33	71	nd	33
Portotur 02	13	15	nd	32	44	nd	nd	nd	104	nd	nd
Elba-30m 01	18	23	10	nd	22	nd	36	nd	73	36	nd
Elba 00	18	23	nd	nd	42	nd	nd	nd	83	nd	nd
Elba 99	15	nd	35	18	26	nd	nd	nd	93	nd	nd
Giglio 99	48	nd	nd	nd	30	3	nd	nd	nd	nd	nd
Procida 00	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Scario 00	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Palinuro 00	nd	nd	nd	35	nd	nd	nd	75	35	nd	75

* Percent w/w with respect to the amount of total carbohydrates.
† Abbreviations: nd, not detectable; Glu, glucose; Ram, ramnose; Man, mannose; Fuc, fucose; Gal, galactose; Ara, arabinose; Aglu, glucuronic acid; Glm, glucosamine; TN, total neutral carbohydrates; TU, total uronic acids; and TAS, total aminosugars.

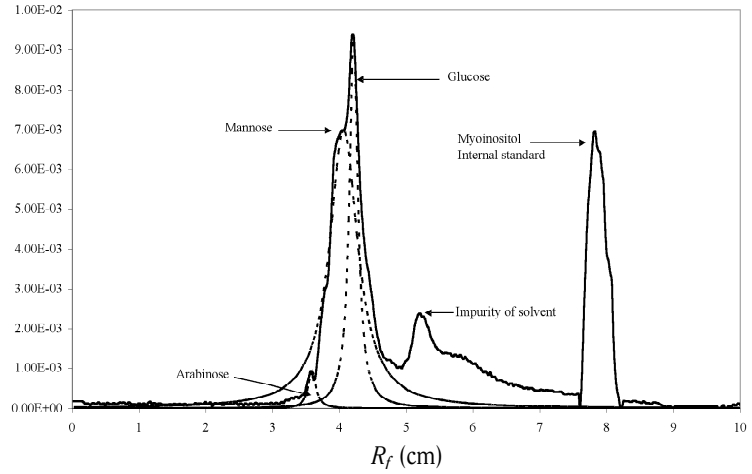

Figure 5. The result of the deconvolution procedure applied to the mucilage sample called Giglio 99. The original chromatogram is reported as a continuous line and the identified peaks as dotted lines.

Table III. Comparison Between the TLC–FID and Colorimetric Methods for the Determination of the Total Carbohydrate Amount on Some Samples*

Samples	% w/w TLC–FID	% w/w Colorimetric
Giglio '99	36.6	30.6
Palinuro '00	2.5	3.2
Elba '99	65.4	71.6
S.F. Circeo-3mt	7.7	4.4
Casaccia	46.27	40.1
Rio Vivo	49.6	44.7

* The amount of carbohydrates refers to the original weight of the sample: correlation coefficient between the two methods, 0.983; equation Y(TLC–FID) = –3.4 + 0.983X (colorimetric); and standard error, 3.99.

Acknowledgments

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