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Rapid microwave-assisted dansylation of biogenic amines Analysis by high-performance liquid chromatography

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Abstract

A fast microwave-assisted dansylation procedure has been developed for the derivatization of biogenic amines prior to HPLC determination. Putrescine, cadaverine, spermidine and spermine are quantitatively dansylated in 5 min using a radiation power of 252 W and a maximum pressure of 3.4 bar inside the reactor. As compared to classical overnight dansylation, the microwave-assisted procedure is 150-times faster. Data concerning repeatability and recovery of method are reported. The method was successfully applied to the determination of the above amines in plant tissues.

Keywords: Microwave-assisted dansylation; Derivatization, LC; Biogenic amines; Putrescine; Cadaverine; Spermidine; Spermine

1. Introduction

Biogenic amines are aliphatic, alicyclic or heterocyclic organic bases of low molecular mass, which arise as a consequence of metabolic processes in animals, plants and microorganisms. Although many of these active substances play normal roles in mammalian physiology, they can also cause unnatural or toxic effects when they are consumed in large amounts [1].

Biologically active amines are normal constituents of many foods and have been found in cheese, sauerkraut, wine and putrid, aged or fermented meat. Their presence in high amounts in foods is associated with food deterioration [2].

The determination of biogenic amines has been carried out with different chromatographic methods: thin-layer chromatography [3–5], gas chromatography [6–8] and high-performance liquid chromatography (HPLC) [9–12].

Biogenic amines are difficult to analyze by a single chromatographic technique because of their structural diversity and lack of an easily detectable common chromophore. The usual approach, therefore, has been to derivatize free amines with an easily detected label group [13].

Three of the more common derivatizing agents used are dansyl chloride [14,15], dabsyl chloride [16] and 7-chloro-4-nitrobenzoxazole (NBD chloride) [17]. Unlike fluorescamine [18] or *o*-phthalaldehyde [19] these have the advantage of forming derivatives with both primary and secondary amines, and all three give derivatives stable enough for subsequent spectroscopy analysis [20,21].

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Dansyl chloride is a particularly well established reagent [22] and gives highly fluorescent sulphonamide derivatives with both primary and secondary amines that are relatively stable, have improved chromatographic properties and are readily isolated from the hydrolysis product, dansyl sulphonate (dansyl-OH), by solvent extraction. Sensitive and selective detection is possible with a fluorescence detector, and dansylated derivatives have also proved useful for spectroscopic identification [23,24].

However, although well established, the dansylation and separation of the amines by HPLC are not particularly suited to automation, because of the time required to carry out dansylation which oscillates between 12 h at room temperature [15,25,26] and 60 min at 37–45°C [9,27,28]. We have therefore developed a new method to reduce the time required to carry out the dansylation of biogenic amines.

The recent application of microwave ovens in chemistry offers a good way for supplying high temperatures in a short time. On the other hand, microwave-assisted procedures can also be employed to accelerate organic reactions [29].

In this work, a rapid microwave-assisted method for dansylation of biogenic amines has been developed to accelerate this step of the biogenic amines analysis. The effect of experimental parameters on the dansylation efficiency has been studied, and the concentration of amine has been determined by HPLC. The method is applied for dansylation and separation of biogenic amines in plant extracts.

2. Experimental

2.1. Chemicals and reagents

Putrescine (Put), cadaverine (Cad), spermidine (Spd), spermine (Spm) and dansyl chloride were supplied by Aldrich (Beerse, Belgium).

For chromatographic analysis, acetonitrile of HPLC grade (Merck) and water purified with a Milli-Q system (Millipore, Bedford, MA, USA) were used throughout.

2.2. Equipment

All measurements were made with a Waters 600 multisolvent delivery system equipped with a U6K sample injector, a Waters Lambda-Max 481 LC variable-wavelength spectrophotometric detector operating at 254 nm and a Waters 740 integrator. The analytical column was a μBondapak C_{18} 10 μm, 100 Å (30 cm×4.6 mm) with a μBondapak C_{18} guard column, both supplied by Waters.

The microwave dansylation system was a CEM (Matthews, NC, USA) MDS 2000 microwave digestion system. The system delivers approximately 630 W (100%) of microwave energy at a frequency of 2450 MHz at full power and provides constant feedback control of reaction conditions through continuous monitoring of pressure data on a control vessel. The system is provided with method and data storage capabilities and a printer.

The microwave dansylation vessels were CEM PFA PTFE-lined advanced composite digestion vessels used for sample dansylation. The vessels are constructed with a PFA PTFE liner and a cap for high-purity analysis that are capable of sustaining temperatures up to 200°C and pressures of 13.9 bar.

2.3. Conventional dansylation method

The dansylated derivatives of the amines were formed by adding to a 2 ml solution of the amine (1 mg/ml), 2 ml of saturated sodium bicarbonate solution, and then adding 3 ml of dansyl chloride reagent (5 mg/ml). The solution was mixed for 1 min and the reaction mixture was allowed to stand overnight. The dansylamines were extracted with two portions of 2 ml of toluene. The extract was taken to 10 ml with toluene. A 100-μl aliquot of the extract was redissolved to 5 ml with acetonitrile. The dansylamines were then quantitatively and qualitatively determined by HPLC.

2.4. Microwave-assisted dansylation method

To a 2 ml solution of the amines, 2 ml of saturated sodium bicarbonate solution and 3 ml of dansyl chloride reagent (5 mg/ml) were added. The solution was mixed for 1 min and transferred quantitatively to microwave dansylation vessels. The vessel was closed and introduced into the microwave cavity. The reaction was made at 40% (252 W) for 5 min, keeping a maximum pressure of 3.4 bar inside the reactor. When the vessel was cold, the mixture was

extracted with two portions of 2 ml of toluene and taken to 10 ml with the solvent. Then we followed the same process as for conventional dansylation.

2.5. Determination of biogenic amines in plant tissue

Tissue extractions were carried out by homogenizing 100 mg/ml of fresh mass tissue in 5% (v/v) $HClO_4$ in an Ultra-Turrax. After 1 h in an ice bath, the extracts were centrifuged for 20 min at 27 000 *g*. The supernatant was derivatized and analyzed as described above.

3. Results and discussion

3.1. Chromatographic conditions

Different solvent mixtures and gradient programmes were tried to reduce the analysis time while keeping a good resolution of all the amine peaks. Good results were obtained using as mobile phase a mixture of acetonitrile and water with a solvent gradient. The solvent gradient procedure employed is reported in Table 1. The different amines gave satisfactory retention times (Fig. 1): putrescine 12.9, cadaverine 15.0, spermidine 21.4 and spermine 24.7.

In order to verify the linearity of the UV detector response at 254 nm for the working concentrations of each amine, a portion of the four amines standard solution was derivatized and injected (10–50 μl) into the HPLC system. Calibration graphs were constructed by plotting the amine peak-area against the amine mass. Data on linearity and detection limits [30] are given in Table 2; a linear relationship with $r>0.99$ was always obtained.

A standard mixture solution was derivatized and injected six times to evaluate the repeatability of the chromatographic system. Means ($\bar{x}$) and relative standard deviations (R.S.D.s) of the response factors are reported in Table 3.

Table 1
HPLC elution programme for amine analysis

Time (min)	Water (%)	Acetonitrile (%)
Initial	40	60
8.0	40	60
13.0	10	90
20.0	10	90
30.0	40	60

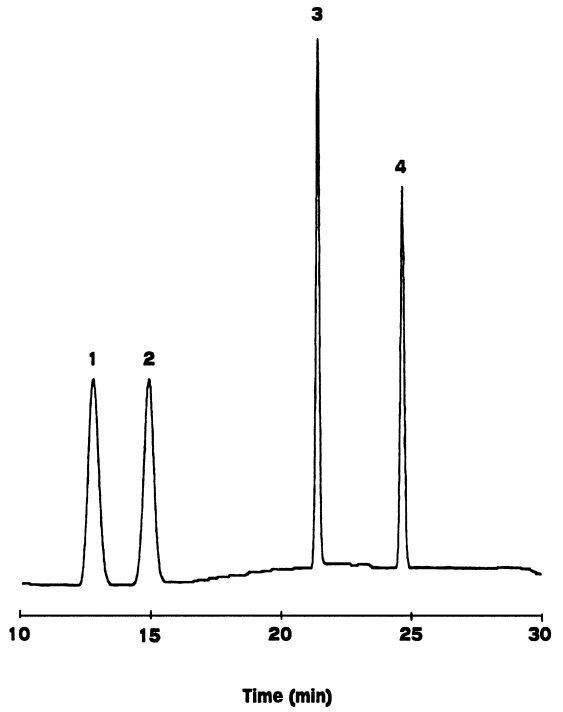

Fig. 1. Optimum chromatographic separation of the dansylated biogenic amines. The amount of each biogenic amine injected was about 60 ng and the flow-rate was 1.2 ml/min. Peaks: 1=putrescine; 2=cadaverine; 3=spermidine; 4=spermine. Experimental conditions are given in Section 3.1.

3.2. Microwave-assisted dansylation of amines

The conventional dansylation of biogenic amines is based on the direct reaction with dansyl chloride, on presence of a high bicarbonate concentration. Numerous authors recommend that the reaction is produced at room temperature during 12 h (overnight reaction).

In order to reduce the time required to reach the dansylation, a series of experiments in closed PTFE reactors was carried out. Among the experimental variables considered, a special attention was paid to the radiation power supplied, pressure inside the reactor and reaction time. The efficiency of the dansylation accomplished in the reactor is established by comparison with the results obtained through conventional dansylation, using in both

Table 2
Detection limit and linearity of the UV detector response at 254 nm

Amine	Detection limit (ng)	(Slope±S.D.)·10^{-3}	(Intercept±S.D.)·10^{-3}	Correlation coefficient
Putrescine	3	5.7±0.2	−20.7±10.0	0.998
Cadaverine	0.6	5.2±0.2	−16.6±8.0	0.999
Spermidine	2	4.8±0.2	−18.1±7.2	0.999
Spermine	3	4.0±0.2	−20.4±8.2	0.998

Linearity range: 0–100 ng.

Table 3
Instrumental repeatability

Amine	Mean	R.S.D. (%)
Putrescine	50.9	1.8
Cadaverine	51.8	4.0
Spermidine	51.3	3.0
Spermine	51.1	3.9

Results are mean of six replicated of the same sample containing 50 ng of each amine.

cases the chromatographic analysis. The studies carried out with different amines showed that the efficiency of the dansylation in the reactor improves upon increasing the power. However, at times equal or higher than 5 min and at high powers (>50–60%), secondary processes are produced that affect the stability of the dansylated derivatives and, consequently, reduce the recoveries. Fig. 2 shows the results of the dansylation of putrescine at different powers.

The pressure and temperature, that are reached inside the reactors, are intimately related to power and time variables. Therefore, in slow kinetic reactions important increases in the reaction rate could be provoked upon increasing the power of the microwave system. The equipment used allows us to establish the relationship between the pressure inside the reactor and the time, for each selected power, as well as to limit the maximum pressure that we want to reach. In this way we have proved that for a power of 40%, the dansylation of the amines takes place with high yields, and in relatively short times, when maximum pressures of about 3.4 bar are used. The use of maximum pressures lower than this lead to a very long time of dansylation, whereas maximum pressures higher lead to low recoveries, possibly by degradation of the dansylated derivative, due to the temperatures reached inside the reactor. Thus, for maximum pressures of 6.9 bar the reached recoveries do not go beyond 25%.

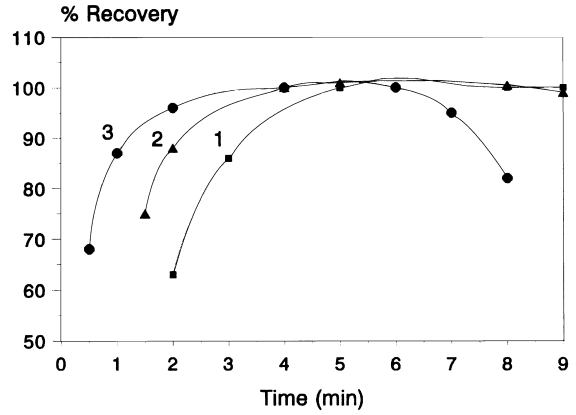

Fig. 2. Effect of the radiation power on the dansylation of putrescine (70 μg/ml). 1=30% (189 W), 2=40% (252 W) and 3=60% (378 W). Maximum pressure: 3.4 bar.

Fig. 3 shows that about 2 min are necessary to reach the pressure of 3.4 bar with a power of 40%. Fig. 3 also shows that, under such conditions, the yield on the dansylation of putrescine is approximately of 90%. To increase this percentage we can increase the power and/or the pressure, with the consequent degradation risk of the dansylated product. In such situation, we have decided on extending the reaction time, after reaching the pressure of 3.4 bar inside the reactor. In this way, with additional times of 2 to 3 min after reaching the maximum pressure of 3.4 bar, dansylations comparable to those which are obtained at room temperature with reaction times of about 12 h are achieved.

Experiences with other powers have been carried out fixing to 3.4 bar the maximum pressure to obtain inside the reactor. Table 4 shows the obtained results, where it is observed that for the studied powers, additional times of 2–3 min after reaching the pressure of 3.4 bar lead to quantitative dansyla-

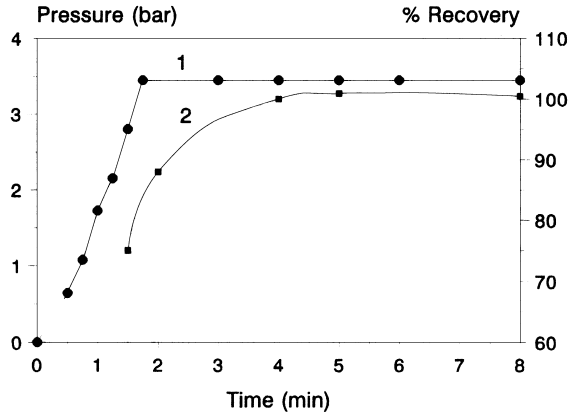

Fig. 3. (1) Pressure versus time data for microwave program. Power: 40% (252 W) and maximum pressure: 3.4 bar. (2) Effect of the digestion time on the dansylation efficiency for an applied power of 40% (252 W). $C_{Put}=70$ μg/ml.

tions. The necessary total times oscillate between 10.5 and 4 min for powers included between 20% and 60%.

The data mentioned above refer to experiments carried out with a single reactor. When several dansylations are carried out simultaneously, the experimental conditions can be maintained: 40% and 3.4 bar of maximum pressure. In this case, the reaction time have to be prolonged until 10 min with six reactors and until 25 min with 10 reactors. In this case, the reaction times can be reduced using higher powers, which allows one to reach 3.4 bar in less time.

Studies of comparison of the two procedures, conventional and microwave dansylation, have been carried out with 11 samples, which contained 50 ng of each one of the amines. For a confidence level of 95%, the *t*- and *F*-test show that there are no statistically significant differences neither between the arithmetic means nor between the variances obtained with both methods of dansylation.

Table 4
Dansylation efficiency of putrescine for different powers

Power (%)	$Time_{total}$ (min)	Recoveries (%)
20	10.5	99
30	6.5	96
40	5.0	101
60	4.0	102

Measurements were carried out 3 min after reaching the pressure of 3.4 bar inside the reactor.
Results are mean of three replicated of the same sample.

Table 5
Recoveries of biogenic amines after microwave dansylation

	Added (ng)	
	18	36
Putrescine	101	96
Cadaverine	99	96
Spermidine	98	100
Spermine	102	100

Mean of three determinations.

On the other hand, for the dansylation of samples containing 18 and 36 ng of the four amines studied, we obtained recoveries ranging from 95.55% to 101.78%. The dansylations of samples containing various ratios of these amines show good recovery data. Results found are summarized in Tables 5 and 6.

3.3. Determination of biogenic amines in plant tissues

In order to test the applicability of the method established in complex matrices, we used plant tissue, because the separation and quantitation by HPLC of derivatives of biogenic amines have been widely adopted for plant studies.

The used biological material is fruit tissue of the banana tree (*Musa acuminata Colla* AAA cv. *Dwarf cavendish*), which was grown in ''vitro'' under a Murashige and Skoog's medium [31] and was subjected to different treatments, either with or without growth regulator or in presence of inhibitors of the polyamines biosynthesis.

Table 7 shows the results found and the great concordance that exists between the obtained values

Table 6
Recoveries of different ratios of amines

Put:Cad:Spd:Spm	Put	Cad	Spd	Spm
4:4:4:1	97	99	92	99
4:4:1:4	99	100	88	88
4:1:4:4	95	98	88	88
1:4:4:4	108	90	88	88

Mean of three determinations. The samples contain 12.5 or 50 ng of each amine.

Table 7
Determination of biogenic amines in plant tissues

	Sample I		Sample II	
	MAD	OND	MAD	OND
Putrescine	157	149	215	212
Cadaverine	2.8	3.2	7.7	10.3
Spermidine	90	83	87	90
Spermine	12	16	9.9	9.3

Values are given as μg/g of fresh mass.
Mean of three determinations. MAD = microwave-assisted dansylation method. OND = overnight dansylation method.

by the classical overnight dansylation and by the microwave-assisted procedure. Fig. 4 shows the chromatogram of one of the samples obtained after microwave-assisted dansylation. The low values of cadaverine and spermine in the studied samples made necessary carry out their determination by the standard additions method.

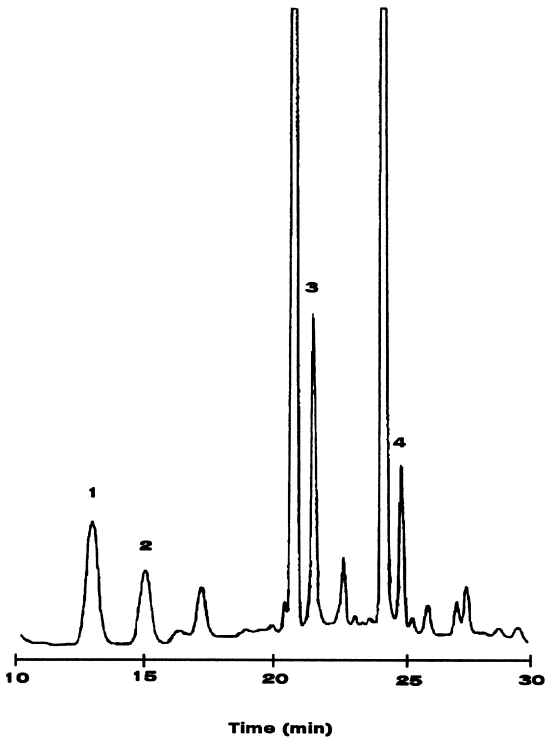

Fig. 4. Chromatogram of a fortified banana tissue analyzed after microwave-assisted dansylation. Peaks: 1 = putrescine; 2 = cadaverine; 3 = spermidine; 4 = spermine.

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