Purification of plant polyamines with anion-exchange column clean-up prior to high-performance liquid chromatographic analysis

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Abstract

Plant material contains carbohydrates and phenolic compounds that interfere with derivatization and HPLC analysis of polyamines. The use of strong anion-exchange resins was investigated for purification of polyamines in plant samples. Results obtained indicate that anion-exchange resins produce equally good results as cation-exchange resins. Polyamines are eluted from the anion-exchange resin with NaOH, and derivatization can be performed directly on the eluate, resulting in enormous time savings. Optimum results were achieved with low cross-linkage strong anion-exchange resins and the conditions for maximum recoveries of polyamines are reported.

1. Introduction

Since polyamines are involved in numerous metabolic processes in plants [1], they have to be quantified accurately. For HPLC analysis, pre-column derivatization is commonly used [2–7], being easy and relatively quick to perform. Post- and on-column derivatization was also described [8,9]. Since derivatization reagents do not only react with amines but also with plant constituents such as phenolic compounds, organic acids, and carbohydrates, interferences can arise. To avoid these problems, a sample clean-up is necessary. Sample clean-up for polyamines is normally performed with cation-exchange resins [3,10,11]

and polyamines are eluted with hydrochloric acid. Before derivatization, HCl has to be removed because most derivatization reagents are acid-chlorides, being inhibited by chloride ions. In addition, these reagents only react with polyamines in alkaline medium. The evaporation of HCl in vacuum is a time-consuming and hazardous step since HCl vapor is highly corrosive. Therefore, the use of anion-exchange resins was investigated as an alternative to cation-exchange resins for sample clean-up. Although anion-exchange clean-up has been explored previously [11], the authors did not elaborate on the resins and procedures employed. In this paper, several anion-exchange resins were tested for their efficacy in removing substances interfering with HPLC analysis. In addition, the parameters affecting the recovery of polyamines from anion-exchange resins are described. The developed

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procedure was applied to several plant samples to test the applicability of the clean-up procedure and to compare it to cation-exchange clean-up.

2. Experimental

Polyamine standards (putrescine, spermidine, spermine, and diaminooctane) were purchased from Sigma. Various anion-exchange resins were tested for their efficacy in removing interfering substances and for recoveries of polyamines. Type I resins (trialkyl functionality): Bio-Rad AG1-X4 (200–400 mesh), Duolite A101, Duolite A101D, Duolite A113, Amberlite IRA-402, Amberlite IRN-78, and Dowex 1-X8. Type II resins (alkylol-dialkyl functionality): Duolite A102 and Duolite A116. All resins, apart from Bio-Rad AG1-X4, were of technical grade with a 14–50 mesh and were milled to correspond to a 100–200 mesh. Before use, the resins were converted to the OH⁻ form with 1 M NaOH and washed with deionized water to remove excess NaOH.

Plant material was homogenized in 5% (v/v) cold perchloric acid (100 mg fresh mass per ml PCA), extracted for 1 h on ice, and centrifuged at 4200 g for 25 min at 10°C. An amount of 1 ml of the supernatant was neutralized with 0.2 ml of 5 M NaOH and 0.5 ml loaded onto Poly-Prep columns (Bio-Rad) containing ca. 1.2 ml anion-exchange resin in the OH⁻ form. Polyamines were eluted with 3.5 ml of a 0.01 M NaOH solution. The eluate was collected in vials and mixed with 0.2 ml of 5 M NaOH to ensure sufficient alkalinity for derivatization. Polyamines were benzoylated for HPLC analysis according to Refs. [12,13]. NaCl solution was omitted after incubation. Polyamines were separated by HPLC on a Spherisorb ODS2 reverse-phase C_{18} column (250 × 4.6 mm, 10 μm) at 35°C, equipped with a Waters Resolve C_{18} Guard-Pak precolumn, and detected at 245 nm [3]. Separation was achieved isocratically at 1.7 ml min⁻¹ with 44% solvent A (water) and 56% solvent B (4% tetrahydrofuran, 96% methanol, v/v).

3. Results and discussion

3.1. Elution profile of polyamines from the anion-exchange column

The elution profile of putrescine, spermidine, and spermine from the Bio-Rad AG1-X4 resin is shown in Fig. 1. A similar elution profile is also obtained with higher and lower molarities of the eluent and with the Duolite A101D resin (data not shown). The results indicate that the first 0.5 ml of the effluent does not contain polyamines (the void volume of the column is ca. 0.3 ml). Thereafter, polyamines elute from the column and elution nears completion after 4.5 ml total volume (1 ml sample volume plus 3.5 ml eluent) have been applied to the column. Benzoylation can be carried out directly on the eluate since a reaction volume of up to 5 ml does not affect the derivatization of individual polyamines (ANOVA, $p > 0.05$, $n = 3$). Hence it is not necessary to concentrate the eluate, resulting in considerable time savings.

3.2. Effect of sample pH and flow-rate on polyamine recovery

Experimental results indicate that if 0.12 ml of 5 M NaOH are added to 1 ml of plant extract before loading, the sample pH is neutral and the
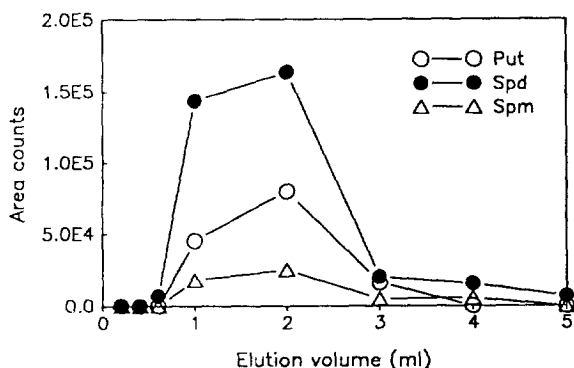

Fig. 1. Elution pattern of putrescine (○), spermidine (●), and spermine (△) from the Bio-Rad AG1-X4 resin in relation to the volume of eluent (1 M NaOH) applied. Elution was performed with gravity flow (1 ml min⁻¹).

recoveries of polyamines are unsatisfactory. Using 0.15–0.2 ml of a 5 M NaOH solution, the sample pH is alkaline and the recoveries of polyamines are higher (Table 1). Therefore, to achieve maximum recovery of polyamines, 0.15–0.2 ml 5 M NaOH should be used to alkalinize the sample extract before loading onto the resin column. An eluent flow-rate of 0.5–2 ml min^{-1} yields constant recoveries of polyamines. Lower recoveries were only observed at a flow-rate of 0.25 ml min^{-1} for spermine (70%), diaminooctane (64%), and spermine (50%), whereas the recovery of putrescine is independent of the flow-rate. Using gravity flow, the flow-rates are normally between 0.75–1.5 ml min^{-1}, which yields constant recoveries for all polyamines. The decreased recovery at low flow-rates is probably due to diffusion and trapping of spermidine, diaminooctane, and spermine in the three-dimensional network of the resin. Also, non-specific retention of polyamines by the anion-exchange column can occur. Thus, to ensure adequate flow, the column should not be packed too tightly.

3.3. Recovery of polyamine standards from various resins

Different anion-exchange resins were tested for the recovery of polyamine standards. Elution was performed with various molarities of NaOH solutions: 2 M (pH 14.3), 1 M (pH 14), 0.1 M (pH 13), 0.01 M (pH 12), and with water adjusted to pH 8 with NaOH. Type II resins (Duolite A116, Fig. 2g; Duolite A102, Fig. 2h) had lower recoveries, ranging from 20% for spermine to 70% for putrescine. Thus, type II resins cannot be recommended for clean-up as recoveries of polyamines are unacceptably low, probably due to hydrogen-bridge bonds between the amino groups of polyamines and the alkylol group of the resin. Type I resins had higher recoveries, of up to 100% for putrescine (Figs. 2a–d), especially at lower pH (pH 8, pH 12). The Bio-Rad AG1-X4 (Fig. 2e) resin has a poor performance in comparison to many cheaper resins. Polyamine recovery from the macropor-

Table 1
Effect of sample pH on recovery of polyamines from the resin

pH of sample	Area counts ($\times 10^6$)		
	Putrescine	Spermidine	Spermine
pH 6–7	0.39 ± 0.07	1.34 ± 0.30	0.16[a]
pH 13–14	0.57 ± 0.04	1.93 ± 0.01	0.30 ± 0.01

Plum buds were homogenized and 1-ml aliquots of the supernatant were mixed with either 0.12 ml 5 M NaOH (corresponding to pH 6–7) or 0.15 ml 5 M NaOH (pH 13–14). An amount of 1 ml of the resulting solutions was loaded onto A101D columns. Results are means of three replicates ± standard deviation.

[a] Based on a single determination only due to co-elution of spermine with impurities in the extract.

ous resin (Duolite A101D, Fig. 2b) is superior to that from the standard type of resin (Duolite A101, Fig. 2f). The high recovery of polyamines from type I resins, especially with weakly alkaline eluents, is possibly due to the fact that the amino groups of the polyamines (pK_a ca. 11) are protonated and therefore repelled by the positively charged functional groups of the resin. Hence, it is suggested that the eluent be adjusted to pH 8–12 for maximum recovery and low cross-linkage or macroporous resins be used. If the eluent is weakly alkaline (e.g. pH 8 or 12), the eluate should be mixed with an additional 0.2 ml of 5 M NaOH. This ensures sufficient alkalinity for the derivatization reaction to proceed optimally. If the molarity of the reaction mixture is below 1 M NaOH, irreproducible results are obtained (data not shown).

3.4. Polyamine concentrations in plant material

Plant material was subjected to purification with Duolite A101D resin as described or benzoylated directly. Values have been corrected for recoveries of polyamines from the resin (putrescine 89%, spermine 89%, diaminooctane 80%, and spermine 95%) and calculated according to Ref. [14]. Alternatively, polyamines were quantified against external standards, corrected for
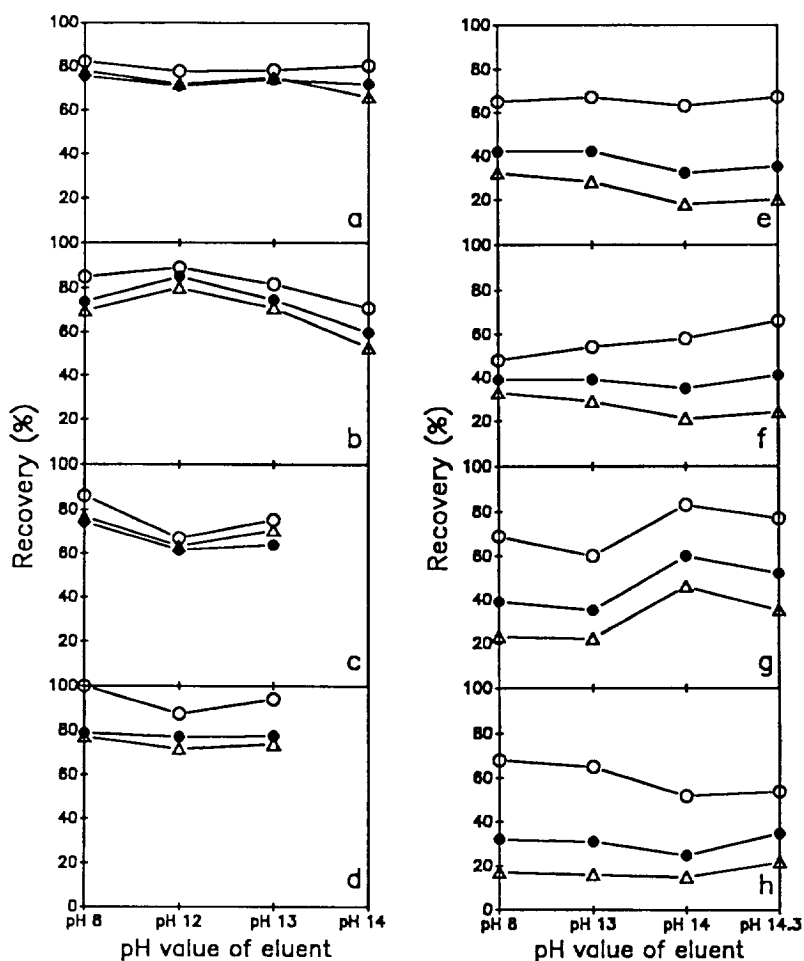
Fig. 2. Recovery of putrescine (○), spermidine (●), and spermine (△) standards from various anion-exchange resins in relation to the pH of the eluent. (a) Duolite A113, (b) Duolite A101D, (c) Amberlite IRN 78, (d) Amberlite IRA 402, (e) Bio-Rad AG1-X4, (f) Duolite A101, (g) Duolite A116, and (h) Duolite A102. Each data point is the average of 2–5 determinations.

recoveries and normalized with the internal standard. Results given in Table 2 indicate that polyamine concentrations can be lower if samples are not purified, and in the case of citrus shoot tips, no peak corresponding to spermine is detected. The recovery of the internal standard (diaminooctane) added to various plant materials ranged between 75–100% (data not shown). It was stated [3] that the benzoylation of polyamines is inhibited by some compounds present in crude plant extracts leading to underestimations of polyamine levels. The authors determined that only 3% of the free polyamines are

recovered as benzoylated polyamines if no cleanup is done. However, other researchers, using different plant materials, determined recoveries of 70–95% even without a clean-up [15–17]. To resolve this discrepancy, plum shoot tip homogenates were also spiked with known amounts of authentic polyamines and the recoveries evaluated. The recoveries were 88% for putrescine, 82% for spermidine, and 67% for spermine for column-purified samples and 108% for putrescine, 90% for spermidine, and 69% for spermine in control samples. Since the recoveries are similar, it can be assumed that substances inhib-

Table 2
Polyamine contents in plant material

		Putrescine[a]	Spermidine[a]	Spermine[a]
Pear shoot tips				
A	Control	31 ± 4	78 ± 7	22 ± 9
	Column	38 ± 2	95 ± 8	19 ± 7
B	Control	21 ± 4	73 ± 7	21 ± 8
	Column	26 ± 2	88 ± 9	18 ± 8
Citrus shoot tips				
A	Control	33 ± 3	38 ± 2	0
	Column	44 ± 2	43 ± 7	4 ± 0
B	Control	37 ± 4	42 ± 4	0
	Column	47 ± 5	47 ± 10	5 ± 0
Apple shoot tips				
A	Control	Not calculated because of co-elution of impurities with DOC		
	Column	116 ± 0	484 ± 21	39 ± 5
B	Control	115 ± 3	476 ± 42	45 ± 7
	Column	119 ± 4	498 ± 38	39 ± 6
Plum shoot tips				
A	Control	103 ± 6	379 ± 17	90 ± 11
	Column	107 ± 4	434 ± 16	102 ± 20
B	Control	100 ± 1	373 ± 36	87 ± 15
	Column	101 ± 3	425 ± 42	99 ± 25

Plant extract was either purified with A101D resin (column) or benzoylated directly (control). Results are means ± standard deviation ($n = 3$). Quantification was performed with either DOC (diaminooctane) as internal standard (A) or against external standards (B).
[a] In nmol g^{-1} fresh mass.

iting the derivatization are apparently not present in plum shoot tips. However, mature Citrus leaves may contain inhibiting factors since measured concentrations are lower for unpurified material. More importantly, no spermine could be detected without a sample clean-up, highlighting the necessity of the sample clean-up to obtain meaningful results.

3.5. Qualitative results

In Fig. 3a, a chromatogram of polyamines in plum shoot tips is shown. No clean-up was done and a number of interfering peaks are present that impair quantification of polyamines due to co-elution. By comparison, Fig. 3b shows a HPLC chromatogram of the identical sample purified on Duolite A101D resin and eluted with water adjusted to pH 8.

In Fig. 4a, plum leaf extract was purified with a cation-exchange resin (Dowex 50W-X8) and the polyamines eluted according to Ref. [3]. By contrast, Fig. 4b shows a HPLC chromatogram of the identical sample which was purified on a Duolite A101D resin. The chromatograms in Figs. 3 and 4 show that the clean-up is effective in removing substances that give rise to interfering peaks on HPLC and also that the degree of purification with the anion-exchange column clean-up is similar to cation-exchange clean-up. The purity of the sample is better if the eluent is more alkaline (pH 13 or 14, data not shown). However, since recoveries are lower, it should be determined to what extent the sample needs to be purified to obtain an optimum balance between purity and recovery. Samples being low in interfering substances can be eluted with water adjusted to pH 8 to 12, whereas samples
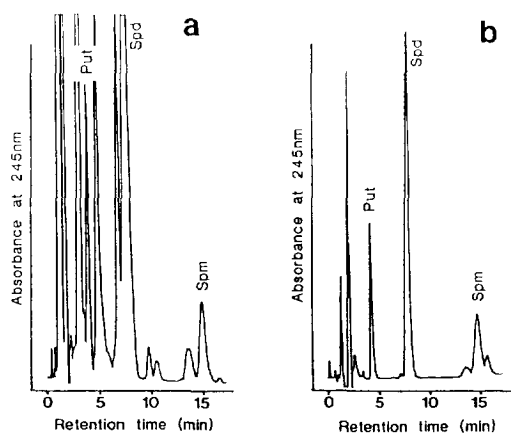
Fig. 3. (a) HPLC-chromatogram of benzoylated polyamines in plum shoot tips. No clean-up was done and a number of interfering peaks are present. The concentrations of the polyamine peaks are: putrescine = 1.4 nmol, spermidine = 3.4 nmol, and spermine = 0.5 nmol. (b) HPLC-chromatogram of plum shoot extract purified with Duolite A101D resin. Polyamines were eluted with water adjusted to pH 8. Concentration of polyamines: putrescine = 0.7 nmol, spermine = 1.7 nmol, and spermidine = 0.3 nmol.

having numerous interfering peaks require a higher molarity NaOH (0.1 or 1 M) to obtain samples of suitable purity.
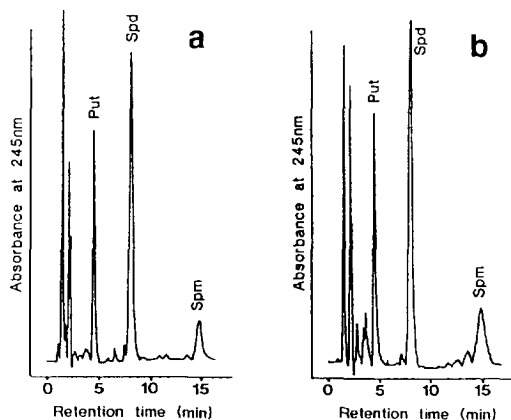

Fig. 4. (a) HPLC-chromatogram of plum leaf extract purified with Dowex 50W-X8 cation-exchange resin. The polyamines were eluted according to Ref. [3]. Concentration of polyamines: putrescine = 0.9 nmol, spermidine = 1.6 nmol, and spermine = 0.2 nmol. (b) HPLC-chromatogram of the identical sample which was purified on a Duolite A101D resin. Concentration of polyamines: putrescine = 1.2 nmol, spermidine = 1.8 nmol, and spermine = 0.4 nmol.

4. Conclusion

Using anion-exchange column clean-up, plant extracts can be successfully purified prior to HPLC analysis. Polyamines can be eluted from the anion-exchange resin without the need to concentrate the eluate, resulting in tremendous time savings. Therefore, the use of anion-exchange resins is a better option and many more samples can be processed per day. In comparison to cation-exchange clean-up, the recoveries of polyamines are similar and the quality of purification is comparable if not superior. The original procedure of Ref. [13] was developed mainly on herbaceous plants that have few interfering substances, unlike woody perennials. However, with the method outlined, it is possible to effectively remove these substances. Furthermore, if the anion-exchange resin is used in the OH⁻ form, no complicating anions are present in the eluate, allowing for simple and rapid derivatization by benzoylation. Phenolic substances, organic acids and carbohydrates [18–21] are optimally retained on anion-exchange resins in alkaline medium. Since polyamines do not carry a net charge in alkaline medium, they are unretained and elute in the void fraction. It should also be determined beforehand whether a sample clean-up is necessary, as an increase in experimental error is normally observed [16] due to the additional experimental variables included.

Acknowledgements

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