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SHORT RESEARCH PAPER

Specific turion yields of different clones of *Spirodela polyrhiza* depend on external phosphate thresholds†

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†In memoriam Elias Landolt (1926–2013)

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ABSTRACT

Turions play an important role in the survival strategy of the duckweed *Spirodela polyrhiza*. Therefore, factors influencing the formation of these survival organs were studied. Phosphate deficiency is the main natural factor inducing turion formation and the specific turion yield (SY), *i.e.* the number of turions formed per frond, varied widely for five different clones from different climate zones. The concentrations of phosphate and nitrate in the nutrient media were investigated at the onset of turion formation, with SY ranging from 0.22 to 5.9. Tissue P and N content was also investigated in vegetative fronds at the onset of turion formation and in newly formed turions. The clones were selected to test possible correlations between SY and threshold nutrient concentration for turion formation. Only one correlation, between SY and external phosphate concentration, was significant: clones with high SY started turion formation at higher external phosphate concentrations. Turion formation is thus mainly induced by the external phosphate concentration, below a defined, clone-dependent threshold. We propose the following mechanism: a switch of the developmental programme of frond primordia from vegetative frond to turion formation at a higher phosphate threshold saves more phosphate for turion formation instead of using it for a further vegetative growth. However, the period of growth preceding turion formation does not depend on this threshold value, but rather on the growth rate of the vegetative fronds, which actually produce the phosphate shortage by taking it up from the surrounding medium.

INTRODUCTION

Species of the duckweed family (Lemnaceae) are distributed throughout the world, being absent only from areas that are too dry (deserts) or too cold (Arctic, Antarctic); they are also very rare where it is too wet (Landolt 1986). Consequently, different duckweed populations have developed diverse ecological strategies to survive unfavourable environmental conditions such as low winter temperatures in temperate climates (Kandeler 1988). Flowering and seed setting is a possible survival strategy (*e.g.* in *Wolffia microscopica*; Khurana *et al.* 1986) but is uncommon in most species, as vegetative propagation dominates within this plant family (Landolt 1986). Over the past 30 years, the authors have observed several overwintering strategies. Some species, *e.g. Lemna trisulca*, become denser and whole fronds sink to the bottom of the water body, to avoid being trapped in surface ice cover. Other species (*e.g. Lemna minor*) overwinter within the surface ice but can regenerate later, mainly due to some meristematic parts that remain intact. Some species form turions (winter buds) that function as survival organs (Jacobs 1947). Turions sink to the bottom of lakes or ponds and in this way survive the low temperatures experienced at the surface. In spring, turions return to the surface (Newton *et al.* 1978) and start a new life cycle involving phytochrome-dependent germination (Appenroth *et al.* 1999).

The developmental steps in the formation and germination of turions are best investigated in *Spirodela polyrhiza*. This species forms so-called 'true turions', which are morphologically different from the leafy fronds and are dormant; they sink to the bottom of the water body and remain dormant until returning to the water surface for germination (Landolt 1986). These properties make them an easily accessible subject for investigation, and thousands of them can be harvested mechanically from the bottom of Erlenmeyer flasks when grown in the laboratory (Appenroth *et al.* 1996). A large number of physical and chemical factors induce turion formation in this species (Appenroth 2002) and have been divided into two classes (Appenroth 2002; and references therein). Factors of class 1 affect the turion number *via* the total yield of the system (exogenously applied sugars, high photon fluence rates or high CO_2 concentrations). Class 2 also contains factors that serve as signals to switch the developmental programme of the primordia directly from vegetative fronds to resting turions (low temperature, low levels of phosphate, nitrate or sulphate, exogenously applied abscisic acid and photomorphogenic effects of light *via* phytochrome or blue light receptors). These factors serve to switch the developmental programme of the frond primordia cells inducing them to produce turions instead of vegetative fronds (Smart *et al.* 1995). Under natural conditions, lower phosphate concentrations at the end of the vegetation

period – caused by P uptake by growing fronds – were shown to be the most important environmental factor (Appenroth & Nickel 2010). In contrast to earlier assumptions (Perry 1968), turion formation is not induced by short-day conditions; *S. polyrhiza* is a day-neutral plant with respect to this developmental process (Appenroth 2003). Decreasing temperature, especially at night, plays an additional role in inducing turion formation (Appenroth & Nickel 2010).

In a previous study, turion formation in 32 clones of *S. polyrhiza* from very different climate conditions was investigated under standardised laboratory conditions. The specific turion yield (SY, number of turions formed per frond) ranged from 0.22 (clone from Cuba) to 5.9 (clone from Albania). It was demonstrated that these differences were caused by an adaptation to different climate conditions (Kuehdorf *et al.* 2013). In order to investigate the turion-inducing mechanism, we selected five of these 32 clones, covering a wide geographic and climatic area, and concurrently determined the phosphate and nitrate concentrations in their growth media at the onset of turion formation, together with the P and N content in both the vegetative fronds and newly-formed turions. This study tested possible correlations between SY and external phosphate or nitrate concentration as well as the internal tissue P or N content in these clones. We also considered whether a high SY was associated with an early start to turion formation.

MATERIAL AND METHODS

Plant material and cultivation

Five clones of *Spirodela polyrhiza* (L.) Schleid. (Table 1) were isolated from a single colony at the beginning of the study; clone 7498 was included as it is the first duckweed clone to have its complete genomic sequence described. The fronds were cultivated in a mineral nutrient medium under axenic conditions, as previously described (Appenroth *et al.* 1996): 8 mM KNO_3, 0.06 mM KH_2PO_4, 1 mM $MgSO_4$, 1 mM $Ca(NO_3)_2$, 5 μM H_3BO_3, 0.4 μM Na_2MoO_4, 13 μM $MnCl_2$, 25 μM Fe(III)NaEDTA. Plant pre-cultivation was carried out in 300 ml Erlenmeyer flasks, each containing 180 ml of nutrient solution, and the temperature was kept at 25 ± 1 °C in all experiments. The cultures were continuously illuminated with white fluorescence light from TLD 18 W/86 tubes (Philips, Eindhoven, The Netherlands). In all experiments, 60 μmol m^{-2} s^{-1} of photosynthetically active radiation was used (*ca.* 9 W m^{-2} total fluence rate; Appenroth 2002) and the irradiance was measured using a LI 250 radiometer (LI-COR; Lincoln, NE, USA). Plants were pre-cultivated for at least 4 weeks, with the complete medium replaced every week. Two, three-frond colonies obtained from this pre-cultivation stage were then used as starting material for the following experiments.

Turion formation

Six samples were examined in two independent experiments, *i.e.* the experiments were repeated twice with three replicates applied, each in different flasks (n = 6) for each of the five clones investigated. Two, three-frond colonies were pre-cultivated as described above. Except for the experiments reported in Fig. 1, plants were inoculated under axenic conditions into 100 ml Erlenmeyer flasks containing 75 ml nutrient media and closed with a cotton-wool stopper. The colonies were maintained under the same light and temperature conditions for 50 days (as per the pre-cultivation) but without changing the nutrient medium. Subsequently, fronds and developing turions were counted. Initial relative growth rates (RGR, day^{-1}) were also investigated using six samples in six different flasks, and were calculated using the number of fronds at the beginning of experiments (N_0, t_0) and 7 days later (N_7, t_7) according to the equation:

$$\mathrm{RGR} = \frac{\ln N_7 - \ln(N_0)}{t_7 - t_0}$$

Thus, the number of fronds was used as a criterion of biomass. The period for the determination of RGR of duckweeds is generally defined as 7 days to avoid growth self-inhibition through overcrowding of the surface by propagating fronds (Naumann *et al.* 2007). The start of turion formation was defined as the time difference between the start of the experiment and the first visible sign of turions. At this stage, the newly formed turions were still attached to the mother plant.

Analytical methods

Aliquots of the growth nutrient media (1 ml) were taken at regular intervals from time zero to day 16 to determine the concentration of phosphate and nitrate during the time-course of plant cultivation and at the onset of turion formation. The decrease in medium volume was the same in all flasks and was not compensated with new medium. At the beginning of turion formation, samples of vegetative fronds and turions were also

Table 1. Basic parameters of growth and turion formation in five clones of *Spirodela polyrhiza*.

clone number (previous term)	origin (year of collection)	SY	RGR (day^{-1})	start of turion formation (days)
9505 (SC, Hav77-S)	Cuba, Havana (1977)	0.22 ± 0.03^{a}	0.453 ± 0.009^{d}	16.0 ± 0.0^{d}
9500 (SJ)	Germany, Jena, Porstendorf (1967)	1.00 ± 0.17^{b}	0.320 ± 0.008^{a}	13.7 ± 0.7^{ac}
7498	USA, North Carolina, Durham (1970)	1.90 ± 0.30^{c}	0.401 ± 0.016^{c}	16.0 ± 0.0^{d}
9256	Finland, Uusimaa, Pukkila (2000)	3.35 ± 0.25^{d}	0.296 ± 0.011^{a}	12.3 ± 0.7^{a}
9501 (Alb06-S)	Albania, Vlora, Matke, 'Keneta e Zeze" (2006)	5.93 ± 0.27^{e}	0.357 ± 0.005^{b}	15.0 ± 0.0^{bc}

SY = specific turion yield; RGR = growth rate. Start of turion formation is the period between the start of the experiment and the first visible signs of turion formation. SY was measured after 50 days and RGR in the first 7 days of the experiments. n = 6, mean ± SE. Letters in brackets show the results of one-way ANOVA (Holm–Sidak test) for each of the three parameters. Different letters indicate significant differences at $P < 0.05$.

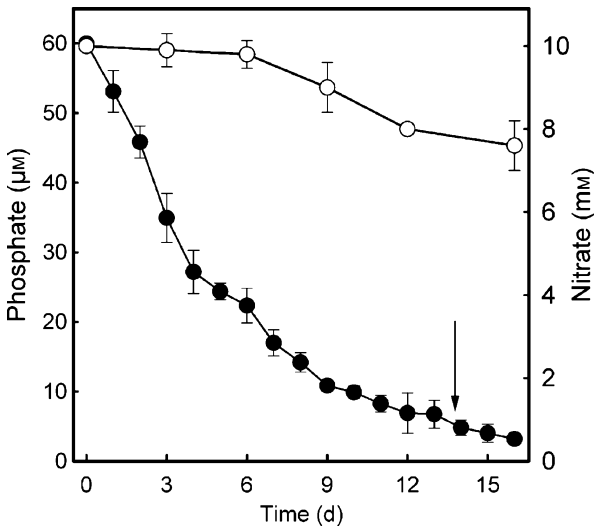

Fig. 1. Decrease in concentrations of phosphate and nitrate during the cultivation of *Spirodela polyrhiza*, clone 9500 under standardised growth conditions. Each culture was started as six colonies with 25 fronds in 180 ml nutrient medium at time zero, and samples from the nutrient medium were taken at the time points .shown. Three independent samples were investigated. Mean ± SE is shown. Closed symbols are phosphate concentration and open symbols are nitrate concentration in the nutrient medium. Arrow: onset of turion formation.

frozen until analysed. In order to obtain control samples from vegetative plants, fronds were cultivated for 7 days under the same cultivation conditions but with the nutrient media changed every day.

The phosphate concentration in the nutrient media was measured using the molybdate method (Worsfold *et al.* 1987); phosphomolybdenum blue was formed and measured at 880 nm using a spectrophotometer. The nitrate concentration of the nutrient media was determined in some samples (data set in Fig. 1) using ion chromatography (DX100 and DX120; Dionex, Idstein, Germany). In other samples (data set in Table 2), the UV direct measurement method was used (Standard Methods for the Examination of Water & Wastewater 1999). Samples were measured at 220 nm and (for correction) at 275 nm in a spectrophotometer (DR 4000 U; Hach, Düsseldorf, Germany).

The dried fronds (105 °C, 2 h; for the same procedure, see Naumann *et al.* 2007) were crushed between forceps and the homogenised dry material was digested and mineralised using concentrated acids before being diluted and analysed for N and P content (for all analytical details, see Adamec 2002). For N analyses, 0.7–0.9 mg dry weight of biomass were mineralised with H_2SO_4 and 1.2–1.8 mg dry weight with $HClO_4$ for P analyses. N and P concentrations were determined colorimetrically using an automatic FIAstar 5010 analyser (Tecator, Hoganas, Sweden). Six independent analyses were conducted for each variant.

Statistical treatment

Six independent samples were examined for each clone. The results for the SY are given as means ± SE, and possible correlations of SY with the phosphate concentration in the external solution at time zero and at the onset of turion formation were calculated. A linear regression model was used to predict SY, with the coefficient of determination (R^2) used as a performance measure.No data were transformed. Significant differences among clones were tested using one-way ANOVA (*xy* test) following the Holm–Sidak procedure.

RESULTS AND DISCUSSION

The concentrations of both nitrate and phosphate in the nutrient media of growing cultures decreased over time (Fig. 1). The growing fronds of *S. polyrhiza* take up these nutrients, creating stress conditions due to partial nutrient shortage, and deficiency of a specific nutrient (phosphate, nitrate or sulphate) represents a signal to induce turion formation (Appenroth *et al.* 1989). In these experiments, this response was observed for clone 9500 13.7 days (on average) after the start of culture

Table 2. Phosphate and nitrate concentration in media, and tissue N and P content in plants of different clones of *Spirodela polyrhiza* at the onset of turion formation.

clone	P_{ext} (µM)	$P_{control}$ (%)	P_{frond} (%)	P_{turion} (%)
9505	2.12 ± 0.94^{a}	0.688 ± 0.021^{ab}	0.0963 ± 0.0062^{a}	0.0530 ± 0.0070^{a}
9500	3.36 ± 1.43^{ab}	0.747 ± 0.018^{bc}	0.173 ± 0.025^{ab}	0.128 ± 0.003^{a}
7498	1.45 ± 0.18^{ab}	0.601 ± 0.027^{a}	0.114 ± 0.008^{ab}	0.0569 ± 0.0036^{a}
9256	6.05 ± 2.69^{ab}	0.794 ± 0.035^{c}	0.141 ± 0.010^{ab}	0.118 ± 0.004^{a}
9501	9.00 ± 0.30^{b}	1.01 ± 0.03^{d}	0.230 ± 0.039^{bc}	0.306 ± 0.042^{b}
clone	N_{ext} (mM)	$N_{control}$ (%)	N_{frond} (%)	N_{turion} (%)
9505	8.47 ± 1.07^{a}	4.36 ± 0.12^{ab}	3.01 ± 0.42^{a}	1.26 ± 0.10^{a}
9500	8.04 ± 0.17^{a}	4.34 ± 0.03^{ab}	3.36 ± 0.31^{a}	1.20 ± 0.10^{a}
7498	7.70 ± 0.10^{a}	4.06 ± 0.07^{a}	2.78 ± 0.20^{a}	1.09 ± 0.09^{a}
9256	9.00 ± 1.18^{a}	4.49 ± 0.04^{b}	2.87 ± 0.14^{a}	1.49 ± 0.07^{a}
9501	8.94 ± 1.00^{a}	4.19 ± 0.10^{ab}	3.18 ± 0.26^{a}	1.05 ± 0.09^{a}

P = phosphate; N = nitrate; ext = concentration in nutrient media at the onset of turion formation; frond = P or N content in vegetative fronds at the onset of turion formation (% of dry weight); control = P or N content in growing control plants; turion = P or N content in turions harvested when still attached to mother plants. Mean ± SE, n = 6. Letters following the data are results of one-way ANOVA (Holm–Sidak). Different letters indicate significant differences at $P < 0.05$.

(Fig. 1, Table 1). The amount of nitrate taken up by growing plants (resulting in a decrease of 2.5 mM in the external medium) was higher than that of phosphate (decrease was 54 μM). However, the concentration of phosphate decreased below 5 μM because of its much lower initial concentration (μM range) in comparison with nitrate (mM range), which remained above 7 mM (Fig. 1, Table 2). As a consequence, turion formation was induced by a shortage of phosphate and not of nitrate. As these experimental nutrient medium compositions often exist in natural water bodies (Lüönd 1983), turion formation in *S. polyrhiza* is induced by a shortage of phosphate under both laboratory (Appenroth *et al.* 1989) and natural conditions (Appenroth & Nickel 2010).

Samples of *S. polyrhiza* collected from different places (ecotypes or clones; Table 1) produced very different numbers of turions per frond (SY) under standardised laboratory conditions. Whereas one clone from Cuba had a SY of 0.22, another clone from Albania produced 5.9 turions per frond. In most cases, the Pearson correlations of SY with all measured parameters resulted in single coefficients of determination (R^2) below 0.10. The coefficients were relatively high for the P content in turions (0.687, $P = 0.083$), in fronds (0.544, $P = 0.155$) at the onset of turion formation, as well as for nitrate concentration in the nutrient media (0.407, $P = 0.247$) at the onset of turion formation. All but one of these correlations was not significant; the only exception was a significant positive correlation between SY and the phosphate concentration in the medium at the onset of turion formation ($R^2 = 0.843$, $P = 0.028$; see Fig. S1).

This significant correlation showed that turion formation is induced primarily by a low level of phosphate in the external medium, which is – at least partly – reflected by decreased P content inside the frond and/or turion tissue. Thus, the external phosphate concentration must be sensed by the plants as a primary signal, while the internal P content shows only a weak correlation and might be considered a secondary endogenous signal. Kufel *et al.* (2012) found that there was no relationship between frond tissue P content and phosphate content in the external medium in one clone of *S. polyrhiza*. In contrast with the present results, however, these authors found that the growth rate of fronds was better described with the Droop model (growth dependent on tissue nutrient content) than with the Monod model (growth dependent on external nutrient availability). A crucial difference between vegetative growth and the induction of turion formation is that in the former case, nitrate and phosphate play a role as macronutrients, whereas in the latter case the (low) concentration of nutrients is used as a signal to switch the developmental programme of the frond primordia to turion formation.

The mean turion P content per dry weight (0.05–0.31%) was at similar level to that in fronds that started forming turions (0.10–0.23%), while the mean turion N content (1.1–1.5%) was about 2.0–2.5 times lower than that in the fronds (2.8–3.4%; Table 2). This indicates that P is much more effectively allocated than N to turions, which serve as storage organs. The external phosphate concentration decreased due to uptake by growing fronds (Fig. 1), which is precisely the mechanism described for turion formation in nature (Appenroth & Nickel 2010). The molecular mechanism underlying the positive correlation between SY of a particular clone and the threshold phosphate concentration of the medium is not known. Turion formation starts with a switch of the developmental programme of frond primordia from forming new vegetative fronds to forming turions (Smart & Fleming 1993), and the existence of a positive correlation means that those clones having a higher SY start turion formation at higher external phosphate concentrations than other clones. Consequently, one may assume that phosphate is not consumed in the formation of vegetative fronds but is available for the formation of a higher number of turions. As shown previously (Appenroth 2002), the total amount of available phosphate in a system influences the number of turions formed.

Apart from the threshold phosphate level, the period required to reach the start of turion formation is another important parameter characteristic of each of the studied clones. A clone with a higher SY, *i.e.* with a higher phosphate threshold level, could be expected to reach that level earlier due to phosphate uptake and would therefore start turion formation sooner. This, however, was evidently not the case (Tables 1 and 2): there was no significant correlation between the time required for each clone to start turion formation and the phosphate threshold level in the medium. Instead, there was a significant positive correlation between growth rates of the clones and the onset of turion formation (Table 1; $R^2 = 0.845$, $P = 0.027$). Therefore, the time to onset of turion formation is determined by the growth rate of vegetative fronds (and thus phosphate uptake rate) and not by the threshold phosphate value.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Correlation between threshold values in the external nutrient medium at which the formation of turions was induced, and the specific turion yield (SY) for five clones of *S. polyrhiza*. The origin of clones and their basic features are given in Tables 1 and 2.

REFERENCES

Adamec L. (2002) Leaf absorption of mineral nutrients in carnivorous plants stimulates root nutrient uptake. *New Phytologist*, **155**, 89–100.

Appenroth K.-J. (2002) Co-action of temperature and phosphate in inducing turion formation in *Spirodela polyrhiza* (Great duckweed). *Plant, Cell & Environment*, **25**, 1079–1085.

Appenroth K.-J. (2003) No photoperiodic control of the formation of turions in eight clones of *Spirodela polyrhiza*. *Journal of Plant Physiology*, **16**, 1329–1334.

Appenroth K.-J., Nickel G. (2010) Induction of turion formation in *Spirodela polyrhiza* under close-to-nature conditions: the environmental signals that induce the developmental process in nature. *Physiologia Plantarum*, **138**, 312–320.

Appenroth K.-J., Hertel W., Jungnickel F., Augsten H. (1989) Influence of nutrient deficiency and light on turion formation in *Spirodela polyrhiza* (L.) Schleiden. *Biochemie und Physiologie der Pflanzen*, **184**, 395–403.

Appenroth K.-J., Teller S., Horn M. (1996) Photophysiology of turion formation and germination in *Spirodela polyrhiza*. *Biologia Plantarum*, **38**, 95–106.

Appenroth K.-J., Gabrys H., Scheuerlein R.W. (1999) Ion antagonism in phytochrome–mediated calcium–dependent germination of turions of *Spirodela polyrhiza* (L.) Schleiden. *Planta*, **208**, 583–587.

Jacobs D.L. (1947) An ecological life history of *Spirodela polyrhiza* (Greater Duckweed) with emphasis on the turion phase. *Ecological Monographs*, **17**, 437–469.

Kandeler R. (1988) Ueberlebenstrategien bei Wasserlinsen. *Biologische Rundschau*, **26**, 347–354.

Khurana J.P., Tamot B.K., Mahshwari S.C. (1986) Induction of flowering in a duckweed, *Wolffia microscopica*, under noninductive long days, by 8-hydroxyquinoline. *Plant and Cell Physiology*, **27**, 373–376.

Kuehdorf K., Jetschke G., Ballani L., Appenroth K.-J. (2013) The clonal dependence of turion formation in the duckweed *Spirodela polyrhiza* – an ecogeographical approach. *Physiologia Plantarum*, **150**, 46–54.

Kufel L., Strzalek M., Wysokinska U., Biardzka E., Okninska S., Rys K. (2012) Growth rate of duckweeds (Lemnaceae) in relation to the internal and ambient nutrient concentrations – testing the Droop and Monod models. *Polish Journal of Ecology*, **60**, 241–249.

Landolt E. (1986) *The family of Lemnaceae – a monographic study. Vol. 1, Biosystematic Investigations in the Family of Duckweeds (Lemnaceae)*. Veröffentlichungen des Geobotanischen Institutes der ETH, Stiftung Rübel, Zürich, Switzerland.

Lüönd A. (1983) *Das Wachstum von Wasserlinsen (Lemnaceae) in Abhängigkeit des Nährstoffangebots, insbesondere Phosphor und Stickstoff. Vol. 3. Biosystematic Investigations in the Family of Duckweeds (Lemnaceae)*. Veröffentlichungen des Geobotanischen Instutes der ETH, Stiftung Rübel, Zürich, Switzerland.

Naumann B., Eberius M., Appenroth K.-J. (2007) Growth rate based dose-response relationships and EC-values of ten heavy metals using the duckweed growth inhibition test (ISO 20079) with *Lemna minor* L. clone St. *Journal of Plant Physiology*, **164**, 1656–1464.

Newton R.J., Shelton D.R., Disharoon S., Duffey J.E. (1978) Turion formation and germination in *Spirodela polyrhiza*. *American Journal of Botany*, **65**, 421–428.

Perry T.O. (1968) Dormancy, turion formation and germination by different clones of *Spirodela polyrhiza*. *Plant Physiology*, **43**, 1866–1869.

Smart C.C., Fleming A.J. (1993) A plant gene with homology to D-myo-inositol-3-phosphate synthase is rapidly and spatially up-regulated during an abscisic-acid-induced morphogenic response in *Spirodela polyrrhiza*. *The Plant Journal*, **4**, 279–293.

Smart C.C., Fleming A.J., Chaloupkova K., Hanke D.E. (1995) The physiological role of abscisic acid in eliciting turion morphogenesis. *Plant Physiology*, **108**, 623–632.

Standard Methods for the Examination of Water and Wastewater (1999) 18th edition, part 4500. American Public Health Association, Washington, D.C., pp 4–87. Available from http://www.standardmethods.org.

Worsfold P.J., Clinch J.R., Casey H. (1987) Spectrophotometric field-monitor for water-quality parameters – The determination of phosphate. *Analytica Chimica Acta*, **197**, 43–50.