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# A rapid and validated HPLC method to quantify sphingosine 1-phosphate in human plasma using solid-phase extraction followed by derivatization with fluorescence detection

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**Abstract**

We describe the development and validation of analytical methodology for the determination of sphingosine 1-phosphate (S1P) in plasma. It uses solid-phase extraction (SPE) followed by an automated reversed-phase gradient HPLC column-switching system with a pre-column derivatization with *o*-phthalaldehyde (OPA) and fluorescence detection. The limit of quantification was determined at 100 ng/ml exogenous sphingosine 1-phosphate with a relative standard deviation for precision and accuracy <15%. The within- and between-day relative standard deviation for precision and accuracy were also less than 15%. This validated method should be suitable to quantify plasma concentration of sphingosine 1-phosphate in relatively large numbers of samples.


*Keywords:* Sphingosine 1-phosphate; SPE; HPLC column-switching; Automated pre-column derivatization; FLU detection; Human plasma

## 1. Introduction

Sphingosine 1-phosphate (S1P) is a sphingolipid metabolite, which can be generated by almost all cell types upon various types of stimulation [1]. S1P can be released from aggregating platelets, and high nanomolar concentrations of S1P are present in human plasma [2,3]. S1P can affect the function of many cell types, mostly as a paracrine mediator, including those of the cardiovascular system [2]. Based upon its wide range of biological effects and its presence in plasma, it has been attempted to correlate plasma concentrations of S1P with biological endpoints such as atherosclerosis. Thus, plasma concentrations of S1P have been reported to correlate with indices of atherosclerosis, even better so than many established indicators of this condition [3]. To further investigate the possible role of S1P in health and disease, it is important to have sensitive, valid and reliable methods for its quantification.

Unfortunately, the existing methods for the measurement of plasma S1P have several important shortcomings. Methods based upon incorporation of radioactive isotopes [4,5] or competitive binding assays [6] lack the ability to resolve structurally related compounds. Alternatively, HPLC methods have been used to quantify plasma S1P because of their ability to resolve a broad range of structurally related compounds. However, the reported methods lack validation [3,7–11] and/or have used internal standards (IS) which also occur endogenously and hence do not allow reliable quantification [8]. Moreover, previously reported methods have used liquid-liquid extraction procedures [7–14] which are time-consuming and do not allow for rapid processing of large numbers of samples. Finally, previously reported S1P measurement techniques require fluorescent derivatization, mostly using *ortho*-phthalaldehyde (OPA), to enhance sensitivity. However, the stability of OPA derivatives varies

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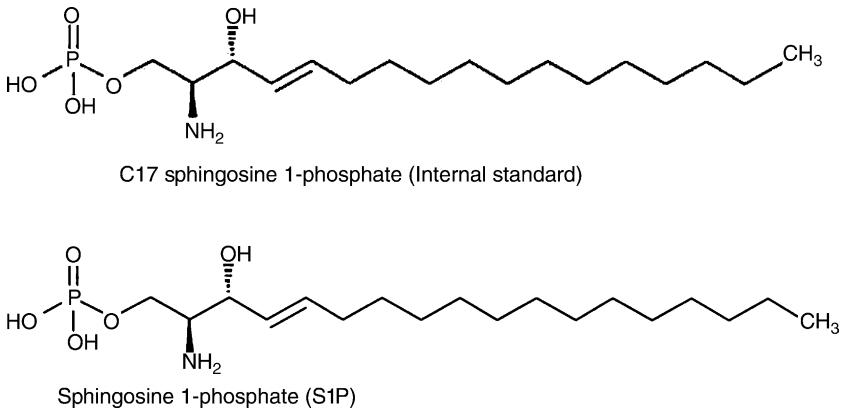


Fig. 1. Chemical structure of sphingosine 1-phosphate and C17-sphingosine 1-phosphate (internal standard).

in time for phosphorylated and non-phosphorylated sphingosines, with the latter being less stable [7]. To limit the impact of such instability upon S1P measurements, an automated pre-column derivatization approach is necessary. Use of analytical mass spectrometry avoids many of the above problems but this has been hampered by high standard deviations [9,10]. While recent improvements in liquid chromatography-tandem mass spectrometry (LC–MS/MS) allow for selective, sensitive and accurate S1P measurement [11,15], they are not available to many investigators due to high hardware costs.

Therefore, we have developed a sensitive and validated method for the quantification of S1P by HPLC, which is suitable for measurements of large numbers of samples. Here we describe a quick and precise method based on a simple solid-phase extraction (SPE) followed by an automated OPA derivatization and reversed phase gradient column-switching HPLC with fluorescence detection. This method was validated using C17-sphingosine phosphate as the IS, as this compound does not exist endogenously.

## 2. Experimental

### 2.1. Materials

(2S, 3R, $4^{E}$)-D-*erythro* SIP and D-*erythro* C17 S1P were purchased from Avanti (Alabaster, AL, USA). o-Phthalaldehyde (OPA) was from Sigma–Aldrich Chemie B.V. (Zwijndrecht, The Netherlands), methanol and acetonitrile (supra gradient) from Biosolve (Valkenswaard, The Netherlands), and boric acid, sodium hydroxide, acetic acid and ammonia 25% from Merck–VWR (Amsterdam, The Netherlands). Solid-phase extraction columns (Waters Oasis HLB), a water-wettable copolymer for reversed phase SPE, 1 cc (30 mg) were from Waters (Etten Leur, The Netherlands). Ultra pure water was prepared by a Milli-Q distillation unit (Millipore, Etten-Leur, The Netherlands).

### 2.2. Reagents and standards

Stock solutions of 200 μg/ml S1P and IS (C17 S1P, Fig. 1) were prepared by dissolving 1 mg in 5 ml chloroform/methanol/ammoniumformate 5 mM (10:10:1). The S1P working solution (10 μg/ml) was obtained by diluting twenty times with methanol/water (1:1) (v/v), and C17 S1P working solution (1000 ng/ml) was obtained by diluting two hundred times with methanol/water (1:1). S1P calibration curves were prepared by spiking 900 μl plasma with 100 μl "10 × calibration concentration". Human plasma for the validation studies was obtained from healthy donors. The OPA reagent contained 10 mg OPA in 5 ml methanol, 10 μl mercaptoethanol and 5 ml 500 mM borate buffer pH 10.4.

### 2.3. Instruments

The HPLC column-switching system consisted of a Triathlon auto injector (Spark Holland, Emmen, The Netherlands) equipped with two electric six-port valves. The valve A (Fig. 2) used for injection was mounted with a 1000 μl straight flush loop. The valve B was used for the column-switching. An isocratic pump (M300, Gyncotec, Germering, Germany) was used for the elution of the pre-column, and a quaternary solvent delivery system (model 1050, Hewlett-Packard, Amstelveen, The Netherlands) equipped with a helium degassing device for the elution of the analytical column. Integration parameters were controlled by PC Integration pack 3.1 (Kontron Instruments-Antec, Leiden, The Netherlands). The short pre-column, mounted in back-flush, for the sample concentration and clean-up was a Hysphere-Resin GP (10 μm, 10 mm × 2 mm, Spark Holland, Emmen, The Netherlands). The analytical column was an Xterra RP18 (5 μm 3.0 mm × 150 mm, Waters, Etten Leur, The Netherlands). The solvent composition for the elution of the short pre-column was 15% acetonitrile in 15 mM phosphate at pH 6.0 with a flow of 1.0 ml/min after injection (Table 1). The mobile phase composition for the gradient system was

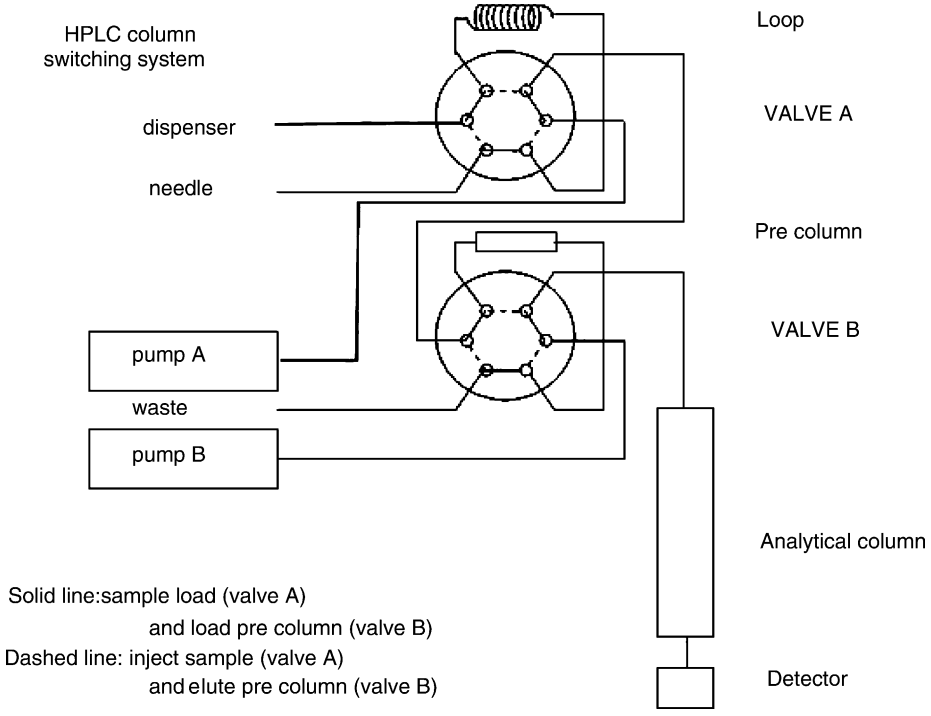


Fig. 2. Flow scheme injection and column switching.

50% acetonitrile in 7 mM potassium phosphate at pH 6.0 for mobile phase A and 100% acetonitrile for mobile phase B. The gradient time program was 0–2 min 100% A, 2–10 min 0–8% B and 10–15 min 8–25% B at a flow of 0.6 ml/min. The detection was performed with a fluorescence detector (model FP 920, 16 μl flow cell, Jasco, Maarsen, The Netherlands) with an emission wavelength of 455 nm and an excitation wavelength of 340 nm.

### 2.4. Sample extraction

SPE columns were conditioned with 2 ml hexane, 1 ml methanol and 1 ml 25% methanol in 0.1% phosphoric acid. Plasma samples of 100 μl were diluted with 100 μl 50% methanol containing IS (100 ng C17-S1P) and thoroughly mixed and further diluted with 0.8 ml 25% methanol in 0.1% phosphoric acid and applied to the SPE column. The SPE column was washed twice with 1 ml 25% methanol in 0.1% phosphoric acid after the sample load and dried in a low vacuum for 5 min. S1P and IS were eluted directly from the SPE column into auto sampler vials with 1.0 ml 90% methanol in borate buffer by a gradient centrifuge step from 0 to 600 rpm in 5 min.

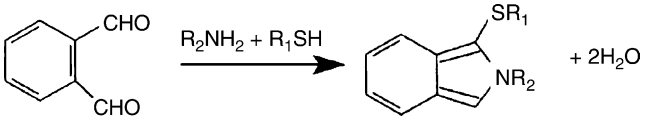


Fig. 3. Reaction scheme for the derivatization of the primary amine with the OPA reagent.

### 2.5. Derivatization

After 1 h saponification at 37 °C, 0.4 ml 10 mM EDTA in borate buffer was added to the sample [7]. The automated OPA derivatization (Fig. 3) was performed with the Triathlon auto injector by adding 20 μl OPA reagent to the auto sampler vial (containing 1.0 ml eluate) and mixing nine times. After incubation for 20 min at room temperature, 500 μl was injected in the HPLC system. The injection cycle started after 5 min.

Table 1
Column-switching events scheme

| Time (min) | Valve B switching configuration | Pump A pre-column (ml/min) | Pump B quaternary system (ml/min) | Event |
|---|---|---|---|---|
| 0–20 | Condition pre-column | 0.1 | 0.6 | Starting cycle (derivatization of sample) |
| 20–25 | Load pre-column | 1.0 | 0.6 | Injection derivatized sample |
| 25–27 | Elute pre-column | 0.1 | 0.6 | Start gradient run (20 min) |
| 27–30 | Condition pre-column | 0.1 | 0.6 | Starting cycle |
| 45–54 | | | | End gradient run, post run (9 min) |

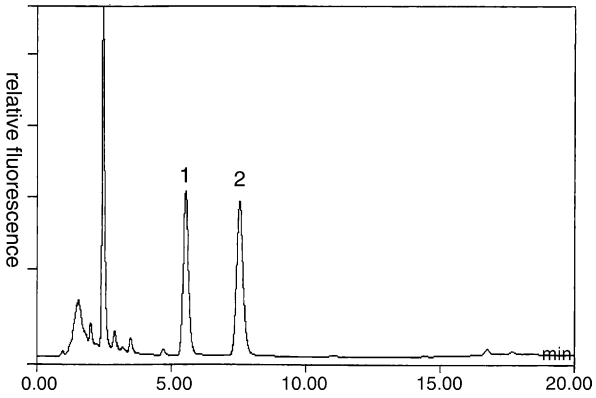


Fig. 4. Chromatogram of a working solution of C17S1P and S1P (1000 ng/ml) derivatized with OPA. The retention times for IS (1) and S1P (2) were 5.4 and 7.2 min, respectively.

## 3. Results

### 3.1. Calibration and recovery

A representative chromatogram for detection of exogenous S1P and IS is shown in Fig. 4. S1P plasma measurements were calibrated according to the standard-addition method (Fig. 5). Thus, S1P concentrations were calculated from a six point calibration line based upon addition of various concentrations of exogenous S1P (50–1000 ng/ml) and a fixed concentration of IS to pooled plasma. The peak area for each S1P concentration was expressed relative to that of the IS, and the resulting ratios were plotted against the nominal S1P concentrations. The data were analyzed by least squares linear regression which typically resulted in regression coefficients of $R^2 = 0.999$. Due to the presence of endogenous S1P in plasma, such regression resulted in negative *x*-axis intercepts, which were used to calculate the concentration of endogenous S1P.

Table 2
Recoveries of S1P and IS

| Compound | Concentration (ng/ml) | Recovery (%) | CV (%) |
|---|---|---|---|
| S1P | 100 | 79 | 11.8 |
| | 500 | 79 | 9.0 |
| | 750 | 77 | 5.4 |
| | 1000 | 75 | 6.5 |
| IS | 1000 | 79 | 5.4 |

Recovery and its coefficient of variation (CV) are based on 6 experiments.

The recovery of extraction of exogenous S1P from plasma was calculated by comparing the peak heights of spiked plasma samples with those of directly injected S1P solution. Recoveries and their coefficients of variation were determined at six different S1P concentrations ranging from 100 to 1000 ng/ml (Table 2), i.e. a range corresponding to previously reported physiological S1P plasma concentrations [3].

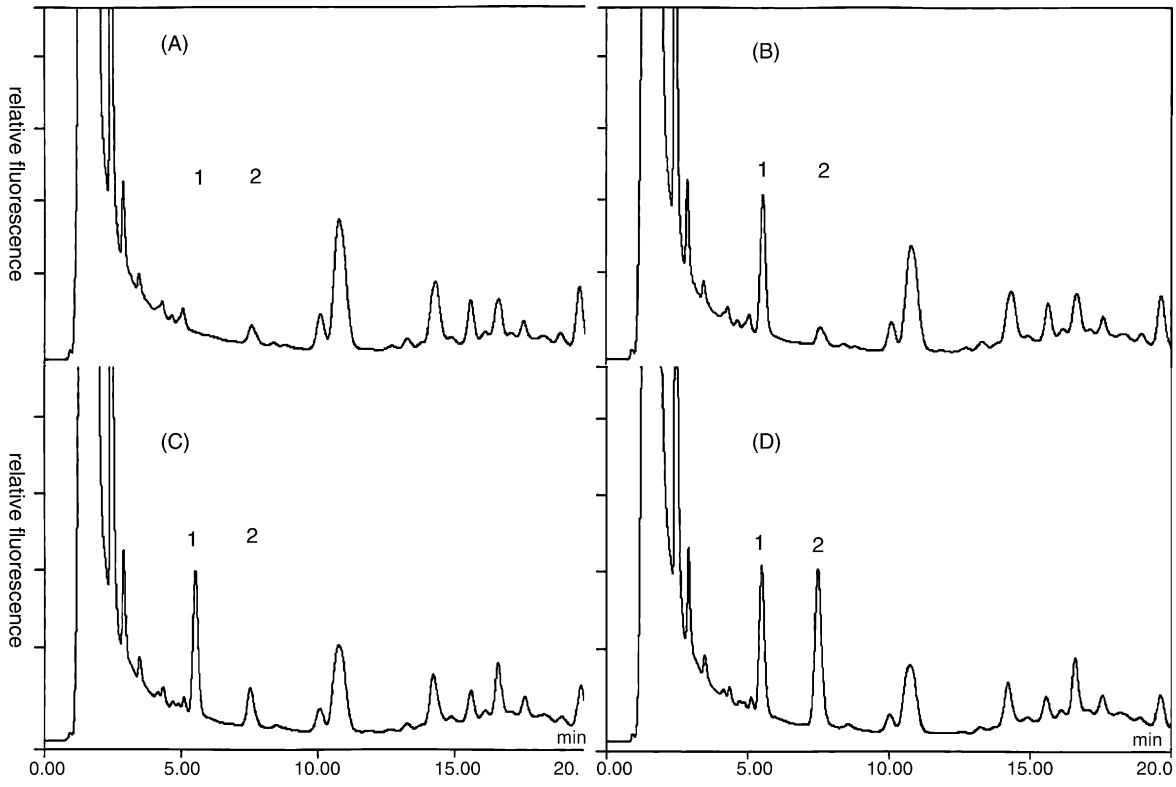


Fig. 5. Chromatogram of a plasma extract of a healthy volunteer in the absence of IS (A), the presence of IS (B), and in the presence of IS and additional presence of 150 and 1000 ng/ml exogenous S1P (C and D, respectively). Within each chromatogram the position of the expected peak for IS and S1P are indicated by 1 and 2, respectively. The endogenous S1P concentration in these chromatograms was determined to be 120 ng/ml.

Table 3
Within-day precision and accuracy of S1P

| Expected (ng/ml) | Found (ng/ml) | CV (%) | Deviation from theory (%) |
|---|---|---|---|
| 100 | 102.4 | 1.8 | 2.4 |
| 250 | 240.7 | 3.4 | −3.7 |
| 500 | 500.8 | 2.4 | 0.2 |
| 750 | 727.3 | 1.3 | −3.0 |

Within-day precision and accuracy are based on six experiments, except for 100 ng/ml where $n = 10$.

Table 4
Between-day precision and accuracy of S1P

| Expected (ng/ml) | Found (ng/ml) | CV (%) | Deviation from theory (%) |
|---|---|---|---|
| 50 | 61.3 | 15.9 | 22.6 |
| 100 | 111.5 | 1.7 | 11.5 |
| 150 | 156.1 | 3.2 | 4.1 |
| 250 | 253.9 | 3.0 | 1.6 |
| 500 | 504.9 | 2.2 | 1.0 |
| 750 | 744.6 | 1.5 | −0.7 |
| 1000 | 1017.0 | 0.7 | 1.7 |

The data are based on measurements on 5 days.

### 3.2. Assay precision and accuracy

Six different pooled plasmas were used for the construction of the validation sample and the calibration line (the same pooled plasma for calibrations and measured validation samples). The lower limit of quantification (LOQ) for exogeneous S1P was determined to be 100 ng/ml (260 nM) with a coefficient of variation (CV%) for precision and accuracy <15% both for within day and between day measurements (Tables 3 and 4).

### 3.3. Chromatography

The elution sequence of IS and S1P followed the expected reversed model with retention times of 5.4 and 7.2 min, respectively (Fig. 4). If required, the retention times of S1P and IS as their OPA derivatives can be tuned by adjusting the pH of the mobile phase. Sphingosylphosphorylcholine, sphinganine 1-phosphate, sphingosine C16 and C20 and sphinganine are endogenous compounds, which have been difficult to separate from S1P by several previously used methods [4–6]. However, they did not interfere with the measurements in concentrations up to 1000 ng/ml because they had clearly different retention times (sphingosylphosphorylcholine 8.4 min, sphinganine 1-phosphate 9.6 min, sphingosine C16 18.5 min, sphingosine C20 > 20 min, and sphinganine > 20 min).

## 4. Discussion

Based upon the growing interest in S1P as an endogenous biomodulator in general and the possible association of S1P plasma levels with disease states in particular, we have developed a validated method to determine S1P concentration in human plasma, which is suitable for assessing large numbers of samples.

Previously published methods in the field have not used an IS [7,9] or used compounds as IS which also exist endogenously, such as sphinganine, and hence cannot be quantified reliably [8]. Based upon a proposal by Min et al. [13], we have used C17 S1P as IS, i.e. a molecule, which has not been detected to occur endogenously. This should offer advantages in the absolute quantification of S1P.

Based upon previously published studies on the presence of S1P in plasma from healthy and diseased subjects, we propose that a clinically useful method for S1P quantification should have a CV of less than 15%. Therefore, we have set this as a threshold to judge the precision and accuracy of our method. In contrast to previously published studies, our data provide a within-day and between-day validation of S1P measurements. Therefore, it was also possible to define a LOQ value for exogenous S1P, which was found to be 100 ng/ml. Such LOQ for exogenous S1P should allow quantification of much lower endogenous concentrations in the standard-addition method. Since endogenous S1P concentrations in plasma and serum have been reported in the high nanomolar range [2,3,16], our method is validated to be sufficiently sensitive to detect endogenous S1P concentrations.

The method described here has several advantages over earlier published methods. Firstly, SPE in combination with pre-column derivatization avoids the time-consuming evaporization, which is necessary with liquid/liquid extraction. Secondly, online pre-column OPA derivatization controls for time dependent instability of the OPA derivatives and should therefore give better reproducibility. Furthermore, it should provide the possibility of a more accurate simultaneous measurement of S1P and other sphinganines as the stability of the OPA derivatives is structure-dependent. Thirdly, our method also has the practical advantage of being quicker than liquid/liquid extraction approaches, which require a time-consuming evaporation step. Since studies on the association between S1P concentrations in plasma and clinical parameters are likely to involve several hundred subjects [3], this expected to enhance the feasibility of clinical studies.

In summary, we have developed an HPLC method for the quantification of S1P in human plasma which allows rapid measurement of absolute quantities in a validated manner. This method should be suitable to quantify plasma concentration of S1P in relatively large numbers of samples as needed for clinical studies.

## References

[1] S. Spiegel, S. Milstien, Nat. Rev. Mol. Cell. Biol. 4 (2003) 397.
[2] A.E. Alewijnse, S.L. Peters, M.C. Michel, Br. J. Pharmacol. 143 (2004) 666.
[3] D.H. Deutschman, J.S. Carstens, R.L. Klepper, W.S. Smith, M.T. Page, T.R. Young, L.A. Gleason, N. Nakajima, R.A. Sabbadini, Am. Heart J. 146 (2003) 62.

[4] Y. Yatomi, F. Ruan, H. Ohta, R.J. Welch, S. Hakomori, Y. Igarashi, Anal. Biochem. 230 (1995) 315.
[5] L.C. Edsall, S. Spiegel, Anal. Biochem. 272 (1999) 80.
[6] N. Murata, K. Sato, J. Kon, H. Tomura, F. Okajima, Anal. Biochem. 282 (2000) 115.
[7] T.B. Caligan, K. Peters, J. Ou, E. Wang, J. Saba, A.H. Merrill, Anal. Biochem. 281 (2000) 36.
[8] L. Ruwisch, M. Schäfer-Korting, B. Kleuser, Naunyn-Schmiedeberg's Arch. Pharmacol. 363 (2001) 358.
[9] P.P. Van Veldhoven, P. de Ceuster, R. Rozenberg, G.P. Mannaerts, E. de Hoffmann, FEBS Lett. (1994) 350.
[10] N. Mano, Y. Oda, K. Yamada, N. Asakawa, K. Katayama, Anal. Biochem. 244 (1997) 291.
[11] B.J. Pettus, J. Bielawski, A.M. Porcelli, D.L. Reames, K.R. Johnson, J. Morrow, C.E. Chalfant, L.M. Obeid, Y.A. Hannun, FASEB J. 17 (2003) 1411.
[12] M.C. Sullards, A.H. Merill, Jr. http://www.stke.org/cgi/content/full/OC_sigtrans;2001/67/pl1.
[13] J.K. Min, H.-S. Yoo, E.-Y. Lee, W.-J. Lee, Y.-M. Lee, Anal. Biochem. 303 (2002) 167.
[14] S.F. Kralik, X. Du, C. Patel, J.P. Walsh, Anal. Biochem. 294 (2001) 190.
[15] M.C. Sullards, Methods Enzymol. 312 (2000) 32.
[16] Y. Yatomi, Y. Igarashi, L. Yang, N. Hisano, R. Qi, N. Asazuma, K. Satoh, Y. Ozaki, S. Kume, J. Biochem. 121 (1997) 969.